REMARKS/ARGUMENTS

Status of the Claims

Claims 1 to 11 were previously presented. Withdrawn claims 8 to 11 are canceled without prejudice. Claims 1 and 7 are amended. No claim amendment is an acquiescence on the merits to a grounds of rejection. Rather, the claim amendments are intended to expedite prosecution of the application. Claims 12 to 26 are newly presented. After entry of these amendments, claims 1 to 7 and 12 to 26 will be pending and presented for examination.

Claims 1 to 7 stand rejected under 35 U.S.C. §112, first paragraph, as not satisfying the written description requirement.

Claims 1 to 7 stand rejected under 35 U.S.C. §112, first paragraph, as not satisfying the enablement requirement.

Claims 1 to 7 stand rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Cryz (Vaccine, 1995), in view of Bukawa (Nature Medicine, 1995) as evidenced by Cryz et al. (Infection and Immunity, 1986).

Support for the amendments to the claims

Claim 1 was amended to set forth a epitope which is not naturally found in the Ib domain of PE. Support for this recital can be found in the specification at page 30, lines 10 and 11, and at page 25, lines 32 to 33. Claim 1 was amended to set forth with more particularity where the epitope is inserted. Support for this subject matter is found in the specification at page 30, lines 10 through 17. Claim 1 was also amended to set forth a fusion protein in sequence. Support for this subject matter is found in the specification in the paragraph bridging pages 27 and 28 and also at page 25, lines 37 to 40. Claim 1 was also amended to set forth a polypeptide which binds a cell surface receptor. Support for this subject matter is found at page 27, lines 12 to 14. Claim 1 was also amended to set forth a different 5 to 350 amino acid size range for the non-epitope domain. Support for this range is found in the specification at page 30, line 20. Claim 1 was also amended to recite 90% sequence identity with respect to the translocation domain sequence. Support for this subject matter is found in the specification at page 16, line 6.

Claim 7 was amended to conform with a change in its antecedent basis.

Support for the epitope domain recitals of claims 12 and 13 can be found in the specification at page 30, lines 18 to 21.

Support for the recital of claim 14 is found throughout the specification, including the Abstract.

Support for the recital of claim 15, is found in the specification at page 28, lines 7 to 11.

Support for the recital of claim 16, can be found in the specification at page 28, line 6.

Support for the recital of claim 17 can be found in the specification at page 4, lines 27 to 30.

Support for the recitals of new claims 18 and 19 can be found inter alia in original claims 8 and 10.

Support for the recitals of new claim 20 is found at page 31, line 29.

Support for the recitals of new claim 21 is found at page 30, line 20.

Support for the recitals of new claim 22 is found at page 16, line 6.

Support for the recitals of new claim 23 is found at page 31, lines 19 to 29.

Claim 24 sets forth a cell surface receptor on the surface of an epithelial cell Support for this recital is found at page 54, line 11.

Support for the recitals of claims 25 and 26 are found in the specification in the paragraph bridging pages 31 and 32.

Support for the recitals of claim 27 can be found throughout the specification. Support for the detoxified domain III is found at page 33, lines 25 and 26.

In view of the above, the Applicants believe the amendments add no new matter and respectfully request their entry.

Response to the rejection of claims 1 to 7 under 35 U.S.C. §112, first paragraph, as allegedly noncompliant with the written description requirement.

As noted by the Office Action, the written description requirement of §112 demands that a specification reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. The Office Action contends that this requirement is not satisfied with regard to cell recognition domains of from 5 to 1500 amino acids in length and epitope domains of from 5 to 1500 amino acids in length.

In one aspect, the Action appeared to be concerned that the Applicants had not shown possession of cell recognition or epitope domains which could be individually re-engineered throughout the stated size range. The Action expressed this concern by stating "It is not known which cell recognition domains and which foreign epitopes will retain the ability to perform the claimed function such as binding to the cell and induction of an immune response, while the amino acid number can vary so drastically" (*see*, Action at page 4, lines 10). The Applicants find this interpretation strained and unlikely to be adopted by one of ordinary skill in the art, especially, in view of the specification at page 27, first paragraph. Rather, as indicated by a fair reading of the specification, the size ranges were intended to set forth a range which would accommodate most such domains of interest, and not to suggest that a given polypeptide needed to be or could be re-engineered to cover the entire range. Indeed, the specification teaches that known polypeptide cell recognition domains and epitope domains can be serviceable. One would reasonably expect that such would come in many sizes from small polpeptide hormones and neurotransmitters to large protein macromolecules, including the many known ligands of the α2-macroglobulin receptor bound by the wild-type PE cell recognition domain. Accordingly, Applicants submit that one of ordinary skill contemplating the broad spectrum of polypeptide ligands for the cell surface receptor would expect the stated size range to be well populated by polypeptide ligands conventional in the art. Nevertheless, the Applicants have amended the base claim to delete the cell recognition domain amino acid length recitals which were a concern.

Next, the Action contends that the Applicants have not adequately described the potential cell recognition domains. While the Applicants note that a variety of cell recognition domain polypeptides are disclosed in the specification at page 27, lines 12 to 24, the Applicants further note that in order to comply with the written description requirement, a specification need not describe what can be readily found in the prior art.

In *Falkner v. Inglis*, No. 05-1324 (Fed. Cir. May 26, 2006) the Federal Circuit ruled that, for claims to nucleic acid sequences and by analogy to amino acid sequences, absence of examples does not render written description inadequate and that actual reduction to practice is not required. *See, e.g., Falkner* slip op at page 14. The court also ruled that publicly available references that describe essential regions of a pox virus could be used to allow those of skill to choose an essential vaccinia gene and then to make a claimed virus. *See, e.g., Falkner* slip op at page 13. Applicants further invite the Examiner's attention to the *Sun* decision by the Board of Patent Appeals and Interferences (see, *Ex parte Sun*, Appeal No. 2003-1993 on Application No. 09/470,526). In *Sun,* the Board considered a claim to a polynucleotide sequence having 80% identity to the entire coding region of a disclosed polynucleotide sequence. The *Sun* Applicants had not disclosed a single representative species with 80% identity and the recited function. The Examiner in *Sun* had argued that the specification did not teach a single variant with the pertinent function. The Board did not find the fact that the specification does not specifically teach the structure of a species with 80% identity and the WEE1 function to be dispositive of the written description question. Rather, the Board looked to the teachings of the prior art and concluded in view of those teachings that one of ordinary skill in the art would have recognized that the Applicants were in possession of the claimed subject matter.

More importantly, with regard to the cell recognition domains themselves, the Applicants note that polypeptides which bind either cell surface receptors and epitopes can only be considered conventional in the art. For instance, the α2-macroglobulin receptor and its ligands have been extensively characterized in terms of their structure and binding properties. For instance, see, Strickland DK et al., FASEB J. 1995 Jul;9(10):890-8 which presents a table listing 19 known α2 macroglobulin receptor binding agents. Thus, Applicants believe many alternative solutions in addition to the particular ones set forth in the specification (use of α2

macroglobulin and anti- α2 macroglobulin antibodies, and Pseudomonas Exotoxin A domain Ia as suitable α2 macroglobulin receptor binding moieties) would exist for a myriad of cell receptor. With regard to epitopes, a PubMed search of published articles reciting the term (see, enclosed print out of first page, turned up over 63000 such articles published through 1996.

In this regard, the Applicants note that the epitope does not lie at the point of novelty in the base claim. With regard to the amount of written description required for inventive features of a claim versus the amount of teaching needed to describe non-inventive features of a claim, there are at least three important court decisions which expressly relax the requirement for non-inventive aspects of patent claims. Of the three cases, *In re Lange*, 209 USPQ 288 (CCPA, 1981), is the most recent and is therefore worth mentioning.

In *Lange*, the invention related to the use of electronegative gases to coat electrical devices to dampen arcing (sparks). The Examiner noted that the claims were broad enough to read on casting of electrodes and that the disclosure was limited to coating of preexisting electrodes. Convinced that this single species was not easily obtainable, the Examiner refused to allow the claims due to over breadth. In rejecting the position of the Patent Office, the CCPA noted that the invention is the use of the gases to dampen sparks. No claim was drawn to casted electrodes. The entire claims were allowed and the CCPA stated:

> However, although appellant can be required to limit his claims to that subject area which is adequately disclosed, the existence of species which are not adequately disclosed does not require that the entire application be found nonenabling. See In re Cook, 58 CCPA 1049, 439, F.2d 730, 169 USPQ 298 (1971). This is especially true in this case where, as stated by appellant at oral argument, the method of forming the electrodes is **not the inventive principle** [Emphasis added].

The other two cases are *In Application of Fuetterer*, 138 USPQ 217 (CCPA 1963) and *Application of Herschler*, 200 USPQ 711 (CCPA 1979). In *Herschler*, the applicant had discovered that dimethylsulfoxide (DMSO) was useful as a transdermal carrier for physiologically active steroids. The CCPA found that a priority application describing a single steroid (dexamethasone 21-phosphate) supported a claim to the genus of all steroids. Citing *Fuetterer*, the court explained that Herschler's claims were not drawn to a novel steroid but to the method of administration of steroids. As long as the class of steroids could be expected to be

carried across the skin by DMSO, the claim could encompass any steroid, known or unknown. As in *Fuetterer*, the CCPA reminded the Patent Office that the inventive principle was a method of administration of steroids and that the specific steroid exemplified was not the point of patentability.

Herschler is particularly on point in the present case. Like *Herschler*, the Applicants chimeric proteins have broad application as carriers of epitopes useful for eliciting a secretory immune response. Here, the Applicants' invention lies in the recited use of the chimeric PE-like protein having an epitope located as set forth in the claims. The novelty of the invention does not lie in the "epitope" itself. In *Herschler*, the invention was a method of passing steroids through the skin, and the claims were appropriately not limited to known steroids. Similarly, the claims of this invention should not be limited to the disclosed epitopes. The fact that unknown, or even known but not fully described, epitopes could be used does not detract from the patentability of the broad claim. This is because the epitope does not constitute the **inventive principle**.

The above precedents are in accord with Example 18 of the PTO's Revised Interim Written Guidelines (enclosed). Example 18 concerns the hypothetical claim:

> 1. A method of producing a protein of interest comprising: obtaining Neurospora crassa mitochondria, transforming said mitochondria with a expression vector comprising a nucleic acid that encodes said protein of interest, expressing said protein in said mitochondria, and recovering said protein of interest.

The hypothetical specification of Example 18 exemplified the expression of beta-galactosidase using the claimed method using a cytochrome oxidase promoter. The PTO's model analysis recognized that the particular nucleic acid was not essential to the claimed invention which lay in the expression system and that the exemplification of a single example sufficed to show possession of the genus.

Having established there is adequate written support individually for the cell recognition domain and epitope domain recitals, the Applicants address the next what may be the major concern of the Action with respect to written description. The Action appears to contend

that one of ordinary skill in the art would not consider that the Applicants were in possession of *operable* chimeric immunogens in the scope claimed. In rebuttal, the Applicants will show that the specification and prior art extensively teach how robust PE is to the modification of its various domains. It is this very robustness which makes PE a desirable platform for molecular engineering.

With respect to the Ib domain, the specification teaches that "[d]omain Ib is not essential for cell binding, translocation, ER retention or ADP ribosylation activity. Therefore, it may be entirely reengineered." (p. 30, lines 24-26). The specification also teaches that "the Ib domain is highly insensitive to mutation. Therefore, although the cysteine-cysteine loop of the native Ib domain has only six amino acids between the cysteine residues, one can insert much longer sequences into the loop without disrupting cell binding, translocation, ER retention or ADP ribosylation activity." (p. 30, lines 4-9).

Applicants provided Examples of inserts having 14 and 26 amino acids for the non-epitope domains. Moreover, the prior art teaches that the PE-like compositions retain their functionality even with very large insertions in the 1b domain. For instance, colleagues of the inventor had previously published a paper showing that Domain Ib can be replaced with the V_H chain of an antibody, which in turn is coupled to the V_L chain of the antibody (Kuan and Pastan, PNAS 93:974-8 (1996), enclosed) to provide a 220 amino acid residue modification which retained the toxic functionality of PE. This example shows that substitutions of a size much larger than the native Ib sequence of about 35 amino acids and on the order of the claimed size range of 5 to 350 amino acids can function.

It should also be pointed out that new dependent claims 12 - 14 relate generally to much smaller sizes for the epitope domain. The specification provides working examples of epitope domains having from 14 and 26 amino acids (p. 47, lines 20-22). Applicants submit that the subject matter of these dependent claims is therefore, in particular, fully supported.

Moreover, the application does not exist in a vacuum. PE was studied extensively in the years preceding the priority date of this application, and persons of skill in the art had available as of the priority date studies in which deletions, substitutions, and other mutations of PE have been made, and in which PE had been coupled to cytokines, antibodies, or antibody

fragments and tested for functionality in killing targeted cells. The present inventors and their collaborators alone published dozens of such studies on these matters prior to the priority date of this application. Attached to this Amendment as evidence thereof are 20 Medline abstracts of articles published just by the inventor or his collaborators between 1990 and the priority date of this application regarding the structure or function of PE and its activity after being fused or conjugated to various cell recognition molecules. These studies show the PE platform to be highly robust to a variety of molecular manipulations, including the addition of large amino acid sequences.

Even with reference to just these 20 articles, persons of skill in the art had at least the following guidance:

- Cytokines and factors such as IL-2, IL-4, IL-6, TGFα, basic fibroblast growth factor and acidic fibroblast growth factor, and transferrin can be used as cell recognition domains to target mutated PEs to target cells (e.g., Heimbrook et al., *PNAS* 87:4697-701 (1990); Beraud et al., *Cell Immunol* 133:379-89 (1991); Batra et al., *Mol Cell Immunol* 11:2200-5 (1991); Pai et al., *Cancer Research* 51(11):2808-12 (1991); Kreitman et al., *Bioconjug. Chem.* 4(6):581-5; Puri et al., *J Immunol.* 152(7):3693-700 (1994); and Siegall et al., *Bioconjug. Chem.* 5(1):77-83). Indeed, more than one cell recognition domain can be attached to the same PE platform in different domains simultaneously and still provide functionality (Heimbrook et al., *PNAS* 87:4697-701 (1990));
- Antibodies RFB4 (an anti-CD22 antibody), MRK16, B3, and B1 can be used successfully as cell recognition moieties to target various PE mutants to target cells (e.g., Mansfield et al., *Bioconjug Chem* 7:557-63;[1] Kuan et al., *Clin Cancer Res* 1:1589-94 (1995); Kuan and Pastan *PNAS* 93:974-8 (1996); Pai et al., *Nat Med* 2:350-3 (1996); Choe et al., *Cancer Res.* 54(13):3460-7 (1994);

[1] The Medline abstracts of the articles from Bioconj Chem provided with this Amendment do not display the year of publication, but according to the search results predate the November 1996 priority date of the present application.

Mickisch et al., *J Urol.* 149(1):174-8 (1993); and Theuer et al., *Cancer Res* 53(2): 340-7 (1993));

- PE with the Ia domain deleted, much of the Ib domain eliminated and with a mAB of about 450 amino acids inserted therein function as immunotoxins. Debinski et al., *Clin. Cancer Research* 1(9):1015 (1995);
- PEs with any of a number of deletions can successfully be used to kill target cells when coupled to the various cell recognition molecules mentioned above. Specifically, the articles set forth no fewer than 7 such mutants, which are referred to in the articles and in the art by their resulting molecular weight in kD: PE33, PE35, PE37, PE38, PE38M, LysPE38, PE40. (See, e.g., Heimbrook et al., supra (re: PE40); Mansfield et al., *Bioconj Chem* 7:557-63 (re: PE35); Kreitman et al., *Bioconj Chem* 3:63-8 (PE38);
- The native C-terminal endoplasmic reticulum retention sequence REDLK of mutant PE35 can be substituted by KDEL (Mansfield et al., *supra*);
- PE4E can have its C-terminal REDLK sequence replaced by KDEL, be fused to a growth factor, and targeted to cells of interest, thus permitting this mutant PE, as well, to be used as a cell recognition moiety of an immunotoxin (Gawlak et al., *Bioconj Chem* 4:483-9), and PE38 can have KDEL replace its C-terminal sequence, be fused to a cytokine, and targeted to cells of interest (Reiter et al., *Int J Cancer*, 58:142-9 (1994));
- PEs with substitution of cysteines for 5 surface residues can be PEGylated and that PEGylated PEs could apparently be translocated to the cytosol (Kuan et al., *J Biol Chem* 269:7610-6 (1994));
- PE37 requires a C-terminal endoplasmic reticulum retention sequence to be cytotoxic, indicating that the toxin may translocate to the cytosol from the ER (Theuer et al., *PNAS* 90:7774-8 (1993));

- The translocation of PE from the extracytosolic compartment to the cytosol is mediated by domain II of PE (Theuer et al., *Biochemistry* 33:5894-900 (1994)).

In addition to these teachings, U.S. Patent 4,892,827, which issued in 1990, teaches the deletion of domain Ia of PE and the covalent bonding of a cell recognition protein to the remaining modified PE. This cell recognition protein can be an antibody, a peptide hormone, a growth factor, and a cytokine. Similarly, U.S. Patent 5,458,878, which issued in 1995, teaches that the cell recognition moiety can be inserted in the C-terminal end of PE. The PE40 mutant, in particular, is taught in U.S. Patent 5,602,095, which is referenced at page 27, line 31 of the present specification and which is incorporated by reference in the present specification. Each of these patents was referenced in the IDS and is already of record in this proceeding.

Thus, in addition to the specification, the practitioner had available the guidance of numerous scientific references and published patents. All of these teachings were available to guide the practitioner as of the priority date of the present application. In view of the extensive experience regarding alterations in PE developed over the years preceding the priority date, those skilled in the art had all the information they needed to make and use the claimed chimeric immunogens.

One of ordinary skill in the art, appreciating all of the above, would certainly contemplate that the inventors were in possession of the claimed invention. Accordingly, the Applicants respectfully request that the above grounds for rejection be reconsidered and withdrawn.

Response to the rejection of claims 1 to 7 under 35 U.S.C. §112, first paragraph, as not satisfying the enablement requirement.

Standard of review

Although not articulated in the Action, whether undue experimentation is required to practice an invention is typically determined by the *Forman* factors. These factors weigh (i) the relative skill of those in the art; (ii) the nature of the invention; (iii) the breadth of the claims; (iv) the amount of guidance presented; (v) the presence of working examples; (vi) the state of the

art; (vii) the predictability of the art; and (viii) the quantity of experimentation necessary. *Ex parte Forman*, 230 U.S.P.Q. 546 (PTO Bd. Pat. App. & Inter. 1986), *In re Wands*, 858 F.2d 731, 8 U.S.P.Q.2d 1400 (Fed. Cir. 1988). The Applicant address each of these factors, particularly as raised by the Action, and thereafter provides a general summary.

(i) Level of Skill in the Art.

Applicants believe that the relative skill and experience of those in the art of is very high as readily evidenced by the authorship of the art disclosed with the previous IDS.

(ii) Nature of the Invention.

The invention is in the relatively complex fields of protein engineering, secretory IgA antibody production, vaccine development, and related therapeutics. However, the Applicants further note that more specifically the subject matter involves the subspecialty of re-engineered PE proteins and their use as carriers.

(iii) Breadth of the Claims.

The claims have been amended in order to expedite prosecution of the application without acquiescing on the merits to a position adopted by the Action. Most notably, the Applicants have amended claim 1 to set forth that 1) the recited domains are listed *in sequence*, 2) a sequence identity of at least 90% for the recited translocation domain sequence, 3) a length of from about 5 to 350 amino acids for the epitope domain, and 4) the epitope domain is inserted directly into the Ib domain of PE, with or without deletion of native Ib amino acid sequence.

(iv) Amount of Guidance Presented, (v) State of the Prior Art and (vi) Working Examples.

The specification and prior art extensively teach how to manipulate and modify each of the various PE domains. Rather than repeat their extensive remarks, the Applicants refer the Examiner to the written description discussion above for a summary of how the specification and prior art teach and exemplify ways to manipulate PE as a platform for molecular transport and/or intracellular trafficking. All the techniques required to practice the invention are exemplified in the specification and are well known in the art. As shown above, the amount of guidance provided in the specification, including working examples, is ample and the state of the prior art can only be described as advanced.

(vii) Unpredictability of the Art.

The field of protein structure and function is generally one in which unpredictability can be an issue and gives rise to the three principal concerns raised by the Action in support of this rejection. The first concern relates to the potential interference of the cell recognition domain upon the immune recognition of the epitope domain. The second concerns relates to the potential effect of the cell recognition domain and the epitope modifications on the ability of the translocation domain and endoplasmic reticulum retention domain to function as set forth in the claims. The third concern relates to whether the intracellular processing of multiple epitopes would interfere with the presentation of the single epitope on the cell surface after processing. The Applicants address each of these issues in turn.

With regard to the potential effect of a cell recognition domain on the recognition of the epitope domain, the Applicants note that the base claim as amended sets forth that the epitope domain is located between the translocation domain and the endoplasmic reticulum retention domain. The cell recognition domain is accordingly far removed in sequence from the epitope domain.

The second concerns relates to the potential effect of the cell recognition domain and the epitope modifications on the ability of the translocation domain and endoplasmic reticulum retention domain to function. This concern is principally addressed by many prior art references as discussed further above and will not be revisited here except to point out that the Cryz et al. references relied upon the Action are themselves in accord with the proposition that the functions of these PE domains are quite robust to modifications:

Cryz et al. 1995, taught a PE molecule covalently modified with the addition of *nine* epitope domains on average, each about 13 amino acids long, and each attached via an adipic acid moiety as linker. Cryz et al., 1986, taught a PE molecule extensively engineered by the attachment of a multitude of PS moieties such that the chimeric immunogen was 27% carbohydrate. Such extensive re-engineering of the cited chimeric construct did not eliminate its activity as a superantigen according to the authors. Moreover, Cryz et al. acknowledged the

overall usefulness of PE by stating "Toxin A was selected since it has been characterized as a superantigen, has been shown to serve effectively as a carrier for other low molecular weight antigens, and is suitable for human use" (see Cryz et al., 1995, page 67, right column, last paragraph before "Materials and Methods").

The third concern relates to whether the intracellular processing of multiple epitopes would interfere with the presentation of the single epitope on the cell surface after processing. This contention appears to be partially inconsistent with the cited Cryz et al. and Bukawa art which each concerned a plurality of epitopes. This concern cites Grigera et al., 1996, as evidencing the uncertainties in making a chimera immunogen by combining epitopes of two immunogens, neither of which was PE, in one protein. In context, this approach to the enablement question appears to suggest that the presence of an inoperative embodiment might serve to negate enablement of an invention. This suggestion, if present, would be wrong. Applicants respectfully note that "[t]he presence of inoperative embodiments does not necessarily render a claim nonenabled." See MPEP §2146.089(b). Indeed, the Federal Circuit has held that "[t]he standard is whether a skilled person could determine which embodiments that were conceived, yet not made, would be inoperative or operative with expenditure of no more effort that is normally required in the art." *See Atlas Powder Co. v. E.I. du Pont de Nemours & Co.*, 750 F.2d 1569, 1577 (Fed. Cir. 1984). If in a given case, multiple epitopes inserted into domain Ib cause a problem, they can be avoided. Accordingly, the Applicants respectfully submit that the instant concern is no bar to enablement.

As discussed at length previously, the prior art available to one of ordinary skill in the art, including that relied upon by the Action, evidences that PE is a robust and readily manipulated carrier which retains its function of translocation and activities even after a heavy dose of re-engineering. This state of the art, when coupled to the Applicant's disclosure, greatly mitigates the traditional uncertainty associated with the protein re-engineering field. PE can only be described as one of the more thoroughly researched, characterized, and modified proteins in biochemistry.

(viii) Undue Experimentation.

The quantity of experimentation necessary[2] to practice the invention with exemplified and non-exemplified embodiments is what is routinely performed by a person of ordinary skill in the art. The specification and prior art provide one of ordinary skill in the art with the general and specific information, moieties, and tools needed to immediately focus their approach so as to minimize the effort needed to practice the invention as claimed. The candidate chimeric proteins can be tested for appropriate trafficking of the chimeric proteins *in vitro* and methods of screening them by testing for the production of secretory IgA antibodies *in vivo* are well known and easily performed as exemplified in the specification. The methods taught to make and screen chimeric proteins are well known in the art.

For instance, a practitioner principally concerned with an epitope of interest can simply use the cell recognition domain of PE and place the epitope in the position set forth in the claims. Or, a practitioner interested in other cell surface receptor targets, would have a large number of alternative cell recognition domains already identified in the specification and in the prior art as likely suitable for use with a PE platform.

Moreover, the therapeutic field of art is traditionally one in which a large volume of screening is both typical and routine. It is a field in which the courts have held that the necessary showing for enablement does not require testing in humans[3].

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[2] That some experimentation may be necessary to identify operative species does not constitute a lack of enablement. As the Federal Circuit has stated, "the key word is 'undue', not 'experimentation' " in determining whether pending claims are enabled. *Wands*, 8 U.S.P.Q.2d at 1405 (Fed. Cir. 1988). Indeed, a considerable amount of experimentation is permissible if it is merely routine, or if the specification in question provides a reasonable amount of guidance for practicing the invention.

3 Usefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans. Were we to require Phase II testing in order to prove utility, the associated costs would prevent many companies from obtaining patent protection on promising new inventions, thereby eliminating an incentive to pursue, through research and development, potential cures in many crucial areas such as the treatment of cancer (In re Brana 34 U.S.P.Q. 2nd 1436 (Fed. Cir. 1995)).
See Scott v. Finney, 34 F.3d 1058, 1063, 32 USPQ2d 1115, 1120 (Fed. Cir. 1994) ("Testing for the full safety and effectiveness of a prosthetic device is more properly left to the Food and Drug Administration (FDA). Title 35 does not demand that such human testing occur within the confines of Patent and Trademark Office (PTO) proceedings.").

(ix) Summary and Overall *Forman/Wands* Analysis.

As set forth in the MPEP §2164.01(a), the final step in making the determination that "undue experimentation" would have been needed to make and use the claimed invention is reached by weighing all the above noted factual considerations. *In re Wands,* 858 F.2d at 737, 8 USPQ2d at 737."

Here, the level of skill for one of ordinary skill in the art is high. Furthermore, the invention is in a field of art which is highly advanced and in which considerable screening is undertaken as a matter of routine. With regard to the breadth of the claims, claim 1 has been amended to address many of the Examiner's concerns. In addition, the Applicant's disclosure, including its working examples, and the prior art as well provide one of ordinary skill all the guidance needed to practice the invention in the scope presently claimed. While the field of protein engineering itself can be in general complex and potentially subject to considerable uncertainty, the subspecialty related to the PE platform itself is quite mature and so extensively characterized that uncertainties related to the PE platform are essentially non-existent given how robust it is to extensive modifications. Moreover, the experimental manipulations involved in practicing the invention are not particularly burdensome and are commonly practiced. Thus, one of ordinary skill in the art would need to do no more than the routine amount of experimentation in order to practice the invention as presently claimed.

Applicants accordingly believe that one of ordinary skill in the art can practice the invention as presently claimed according to the requirements of 35 U.S.C. §112, 1st paragraph and respectfully request that the above rejection be reconsidered and withdrawn.

Response to the rejection of claims 1 to 7 under 35 U.S.C. §103(a) for alleged unpatentability over Cryz (Vaccine, 1995), in view of Bukawa (Nature Medicine, 1995) as evidenced by Cryz et al. (Infection and Immunity, 1986)

In order to establish a *prima facie* case of obviousness, the rejection must demonstrate that (1) the cited references teach all the claimed elements; (2) there is a suggestion

or motivation in the prior art to modify or combine the reference teachings; and (3) there is a reasonable expectation of success. MPEP §2143; *In re Vaeck*,20 USPQ2d 1438 (Fed. Cir. 1991).

Here, the cited art does not disclose all the limitations of the base claim. As amended, claim 1 sets forth that *the amino acid sequence of the epitope domain is inserted directly into the sequence of the Ib domain of PE, with or without deletion of native Ib amino acid sequences;*

Cryz, 1995, in contrast, discloses a conjugate vaccine of Pseudomonas Exotoxin A ("PE") covalently coupled to a HIV-1 polypeptide. Prior to coupling to the HIV-1 polypeptide, the PE was detoxified by covalent coupling to Adipic acid dihydrazide (ADH) in a molar ratio of 10:1. The polypeptide was then coupled to the PE-ADH conjugate via the ADH moiety by use of carbodiimide in a molar ratio to PE of 9:1. The conjugates lacked ADP ribosylation activity and were therefore non-toxic. The conjugates were described as being effective immunogens. Accordingly, Cryz, 1995, does not disclose or suggest a chimeric immunogen wherein *the amino acid sequence of the epitope domain is inserted directly into the sequence of the Ib domain of PE, with or without deletion of native Ib amino acid sequences.*

Cryz, 1986, concerns a polysaccharide moiety covalently coupled to PE-ADH by use of chemical linkers. This reference does not disclose or suggest a chimeric immunogen wherein *the amino acid sequence of the epitope domain is inserted directly into the sequence of the Ib domain of PE, with or without deletion of native Ib amino acid sequences.*

Bukawa, 1995, is alleged to disclose methods directed toward the neutralization of HIV-1 by secretory IgA induced by oral administration. The peptide vaccine candidate used cholera toxin as an adjuvant. This reference also does not disclose or suggest a chimeric immunogen wherein *the amino acid sequence of the epitope domain is inserted directly into the sequence of the Ib domain of PE, with or without deletion of native Ib amino acid sequences.*

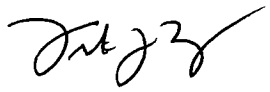
As the cited references do not teach all the claimed elements of the amended claims, either alone or in combination, these references do not establish a *prima facie* case of obviousness. Accordingly, the Applicants respectfully request that the above grounds for rejection be reconsidered and withdrawn.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 925-472-5000.

Respectfully submitted,



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LDL receptor–related protein: a multiligand receptor for lipoprotein and proteinase catabolism

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ABSTRACT **The accumulation of excessive cholesterol–rich lipoproteins within vascular cells, the proliferation of vascular cells, and fibrin deposition are hallmark features of atherosclerosis. Evidence accumulated over the past few years supports the hypothesis that one member of the LDL receptor family, the low density lipoprotein receptor–related protein (LRP), affects the dynamics of each of these processes. LRP is expressed in several vascular cell types, including smooth muscle cells, and in macrophages, and is also expressed in these cells in atherosclerotic lesions. This receptor is a large endocytotic receptor that mediates the catabolism of a number of molecules known to be important in vascular biology, including apolipoprotein E– and lipoprotein lipase–enriched lipoproteins, thrombospondin, and plasminogen activators. The capacity of LRP to mediate lipoprotein catabolism may be a factor in the development of the lesion by contributing to the formation of foam cells. LRP has recently been shown to mediate the catabolism of thrombospondin, a molecule that has potent biological effects on cells of the vasculature. The regulation of its extracellular accumulation by LRP might modulate the dynamic processes of tissue remodeling associated with the response to vascular injury. In addition, LRP regulates the expression of plasmin activity by directly binding and mediating the cellular internalization of urokinase– and tissue–type plasminogen activators. The cellular removal of these two enzymes decreases the local profibrinolytic potential, possibly leading to a thrombotic state at lesion sites.—Strickland, D. K., Kounnas, M. Z., Argraves, W. S. LDL receptor–related protein: a multiligand receptor for lipoprotein and proteinase catabolism.** ***FASEB J.*** **9, 890–898 (1995)**

Key Words: plasminogen activations • thrombospondin • lipoprotein lipase • RAP • chylomicron remnants

PROTEINASES PLAY AN IMPORTANT ROLE IN BIOLOGY, and their activity is carefully regulated by proteinase inhibitors such as α_2–macroglobulin (α_2M)[2]. After reaction with an enzyme, α_2M undergoes a transformation that allows it to bind to cell–surface receptors (1). The existence of a liver receptor responsible for removing α_2M–proteinase complexes from the circulation was first suspected in early studies where it was observed that α_2M–trypsin complexes were rapidly eliminated from the dog circulation. The α_2M receptor was subsequently purified by affinity chromatography (2, 3), and protein sequencing studies revealed it to be identical to the LDL receptor–related protein (LRP) (4).

LRP was initially identified when a cDNA was cloned by a homology screening approach (5). Because the encoded protein resembled the LDL receptor and was able to bind apoE–enriched lipoproteins (6), LRP was suspected of being responsible for the catabolism of lipoprotein "remnants." These particles are generated from two key lipoproteins, chylomicrons and very low density lipoproteins (VLDLs), which are responsible for transporting triglycerides and other lipids from the intestine or liver to other tissues, such as muscle. During this process, chylomicrons or VLDLs are modified by hydrolases, such as lipoprotein lipase, to form remnants, which are then catabolized by a receptor (or receptors) present in the liver. Even though the LDL receptor is able to mediate the clearance of remnants, genetic evidence (7) suggests that another liver receptor is also involved in this process, and available evidence suggests that LRP may be this receptor.

It is now apparent that LRP is responsible for mediating the cellular internalization of many ligands, including proteinases, proteinase–inhibitor complexes, lipoproteins, a bacterial toxin, and a minor–group human rhinovirus. In addition to its role in the hepatic removal of several ligands from the plasma, LRP is also essential for embryonic development (8). This review will summarize the structure of LRP, its role in the catabolism of lipoproteins and proteinases, and its potential role in pathophysiological processes.

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[2]Abbreviations: α_2M, alpha$_2$–macroglobulin; LRP, LDL receptor–related protein; VLDL, very low density lipoprotein; RAP, receptor–associated protein; α_2M*, α_2M–proteinase complex; tPA, tissue–type plasminogen activator; uPA, urinary–type plasminogen activator; uPAR, uPA receptor; TSP, thrombospondin; gp330, glycoprotein 330; β–VLDL, β–migrating very low density lipoprotein; PAI–1, plasminogen activator inhibitor type 1.

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THE LDL RECEPTOR FAMILY

LRP is a member of the LDL receptor family. The first member of this family to be characterized was the LDL receptor, which plays a key role in cholesterol homeostasis (9) by mediating the cellular internalization of apolipoprotein B and/or apolipoprotein E (apoE) containing lipoproteins. The proposed domain organization of the LDL receptor is shown in **Fig. 1**, along with other members of this receptor family. The LDL receptor has a 50 residue cytoplasmic domain which contains an NPXY sequence that targets this receptor to clathrin–coated pits (9). The extracellular portion contains an O–linked sugar domain and two clusters of cysteine–rich repeats. The first cysteine–rich cluster has homology with the epidermal growth factor precursor and contains three epidermal growth factorlike repeats that are separated by five copies of a repeat, each containing a common tetrapeptide, tyrosine–tryptophan–threonine–aspartic acid. This epidermal growth factor homology region appears necessary for the LDL receptor to undergo an acid–dependent conformational change that releases ligands within the endosomes, allowing unoccupied receptors to recycle back to the cell surface (9). The second cysteine–rich cluster contains seven complementlike repeats, which are responsible for binding the ligands apolipoproteins B and E (9).

The most recently discovered member of the LDL receptor family is the very low density lipoprotein (VLDL) receptor. This receptor was so named because it was found to bind apoE–containing lipoproteins such as VLDL with high affinity, but with weaker affinity to LDL (10). The primary sequence and gene structure of this receptor is similar to that of the LDL receptor, except that the VLDL receptor gene contains an extra exon that encodes an additional cysteine–rich repeat sequence within the ligand binding domain (11). A cDNA encoding a variant form of the VLDL receptor that lacks the O–linked sugar domain has also been identified (11). The VLDL receptor, like LRP (see below), is highly conserved between various species. Chickens express a VLDL receptor similar in structure to the mammalian receptor (12). Insight into the function of the avian receptor has been obtained by identifying a mutant hen that lacks the receptor and is characterized by hereditary hyperlipidemia and the absence of egg laying (13). Thus, the chicken VLDL receptor plays a critical role in mediating the transport of triglycerides into growing oocytes (13). Whether the role of the VLDL receptor extends beyond lipoprotein catabolism is not known at this time. Like LRP and gp330, ligand binding by the VLDL receptor is modulated by the 39 kDa receptor–associated protein (RAP; see below) (14).

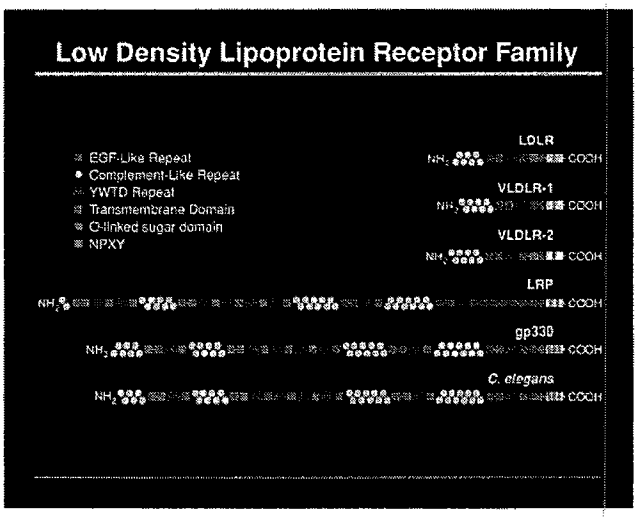

Figure 1. Proposed domain organization of members of the LDL receptor family.

TABLE 1. Ligands for LRP

Proteinases and inhibitor complexes
α_2M-proteinase complexes
PZP-proteinase complexes
t-PA
u-PA
t-PA:PAI-1
u-PA:PAI-1
uPA:protease nexin 1
Tissue factor pathway inhibitor
Elastase-α_1-antitrypsin
Lipoproteins
Apo E
Apo E-enriched β-VLDL
Lipoprotein lipase
Lipoprotein lipase-enriched VLDL
Lipoprotein lipase-enriched β-VLDL
Hepatic lipase
Matrix proteins
Thrombospondin
Other molecules
Pseudomonas exotoxin A
Lactoferrin
RAP

LRP is considerably larger than either the LDL receptor or the VLDL receptor. The LRP cytoplasmic domain contains 100 amino acid residues with two copies of the NPXY sequence. The O–linked sugar domain of the LDL receptor is replaced by six growth factorlike repeats in LRP. LRP contains a total of 22 growth factor repeats and 31 complement–type repeats arranged into four clusters, and is synthesized as a single–chain precursor that is cleaved in the *trans* Golgi to a 515 kDa heavy chain and an 85 kDa light chain (15). The larger subunit, which contains the ligand binding regions, associates noncovalently with the smaller subunit, which contains the transmembrane domain. LRP, which is highly conserved in different species, binds numerous ligands (**Table 1**) and mediates the cellular internalization of proteinases, proteinase–inhibitor complexes, and certain apoE– and lipoprotein lipase–enriched lipoproteins.

The fourth member of the LDL receptor family is a molecule termed glycoprotein 330 (gp330). The overall size and domain organization of gp330 are similar to that of LRP (16). The cytoplasmic tail contains 213 amino acid residues, with three copies of the NPXY sequence. The extracellular domain contains 36 complement–type repeats, forming four clusters of putative ligand binding domains, and 16 growth factor repeats. The same arrangement of modules is conserved in a gp330–like protein whose gene was recently identified in *Caenorhabditis elegans* (17). Although the function of gp330 is not known at this time, this receptor binds many (but not all) of the ligands that interact with LRP. The distinct distribution of gp330 on absorptive epithelial cells suggests a unique role for this receptor.

RAP IS AN ANTAGONIST OF THE LDL RECEPTOR FAMILY

During the course of purifying LRP by ligand affinity chromatography, a 39 kDa protein termed the receptor–associated protein (RAP) was identified (18). RAP binds with high affinity to LRP (K_D = 4 nM) and antagonizes the ligand binding properties of this receptor, preventing it from mediating the cellular internalization of ligands (19). LRP contains multiple ligand binding sites, each independently regulated by RAP (19). RAP also binds with high affinity to gp330 (K_D = 8 nM) (20) and the VLDL receptor (K_D = 0.7 nM) (14), but with much lower affinity to the LDL receptor (K_D = 500 nM) (21), and antagonizes the ligand binding properties of these receptors as well.

The function of RAP and the significance of its interaction with members of the LDL receptor family are not completely understood at this time. It is clear that exogenously added RAP (19) or overexpression of RAP (22) both inhibit LRP activity, and thus concepts regarding the function of RAP center around its ability to block ligand binding by members of the LDL receptor family. If the in vivo function of RAP is to modulate ligand binding by members of the LDL receptor family, then the details of how this occurs are not known. This is because the cellular localization of LRP and RAP differ. RAP is an intracellular protein, found primarily within the endoplasmic reticulum (23), whereas LRP is primarily found within endosomal vesicles or on the cell surface. Thus, these two molecules are contained within different cellular compartments. The localization of RAP within the endoplasmic reticulum suggests that it may play an important role in the early processing of these receptors, perhaps in regulating receptor transport or trafficking to the cell surface. It is interesting to point out that RAP shares functional features with an intracellular chaperone, termed the invariant chain (24). The invariant chain associates with newly synthesized MHC class II molecules, prevents them from binding ligands (in this case antigenic peptides), and appears to be important for targeting class II molecules to their destinations within the endosomes (24). Like the invariant chain, RAP may assist in intracellular trafficking of some of the LDL receptor family members.

ROLE OF LRP IN LIPOPROTEIN CATABOLISM

Overview

Chylomicrons function to transport dietary fat and cholesterol from the intestine through the lymph into the blood and are metabolized in multiple steps. First, most of the triglycerides are removed in a process catalyzed by lipoprotein lipase, a heparin–binding enzyme. The smaller, cholesterol–rich remnant particles are then removed from the circulation by liver parenchymal cells. This process is thought to occur by an initial interaction of the lipoproteins with cell–surface proteoglycans, followed by internalization via a receptor–mediated process (25). Efficient catabolism of lipoproteins requires apoE to be present on the particle surface and can be mediated by the LDL receptor. However, because these lipoproteins are efficiently catabolized in homozygous familial hypercholesterolemia patients (7) who have severe deficiencies of LDL receptors, another molecule, perhaps LRP, must assume this function in these patients.

Secretion–recapture model for remnant metabolism

Several lines of evidence support a role for LRP as a chylomicron remnant receptor. As a model lipoprotein to study chylomicron remnant catabolism, β–migrating very low density lipoprotein particles (β–VLDL) have been utilized. These lipoprotein particles accumulate in the plasma of cholesterol–fed rabbits. The isolated lipoproteins do not bind to LRP. However, when they are incubated with additional apoE they are then recognized by LRP. This has been demonstrated in ligand blotting experiments where apoE–enriched β–VLDL particles are able to bind directly to LRP present in rat liver membrane extracts (26). A role for LRP in mediating the internalization of these lipoproteins was demonstrated when β–VLDL, enriched with exogenously added apoE, stimulated incorporation of [^{14}C]oleate into cholesterol [^{14}C]oleate in FH fibroblasts (6). This sensitive assay measures receptor–mediated uptake of cholesterol–rich particles. Because the stimulation was blocked by an antibody against LRP (6), these studies suggest that LRP mediates the uptake of chylomicron remnantlike particles, but only after they have been enriched by excess apoE. This requirement for excess apoE led to a secretion–recapture model for remnant uptake in the liver (25, 27). In this model, remnant particles are envisioned to enter the sinusoidal space of the liver. The remnant particles are thought to adhere to extracellular proteoglycans, and are exposed to high concentrations of apoE secreted by hepatocytes. After enrichment of the remnants with apoE, they are active ligands for LRP, which can then mediate their internalization and subsequent degradation.

Role of lipoprotein lipase in LRP–mediated lipoprotein catabolism

Beisiegel et al. (28) demonstrated that lipoprotein lipase enhances the binding of chylomicron remnants to fi-

broblasts and that lipoprotein lipase can be cross–linked to LRP. These observations led to the hypothesis that lipoprotein lipase may promote the internalization and degradation of lipoproteins. This hypothesis is supported by data provided by Chappell et al. (29) who observed that although VLDL itself does not bind to LRP, lipoprotein lipase promotes the binding of VLDL to purified LRP and promotes the internalization and degradation of VLDL in fibroblasts deficient in the LDL receptor (29). The degradation of these enriched lipoproteins is blocked by LRP antibodies, indicating that LRP is the receptor responsible for their cellular internalization. Thus, lipoprotein lipase may directly facilitate the catabolism of lipoproteins by promoting their cellular internalization by LRP.

The ability of lipoprotein lipase to promote LRP–mediated lipoprotein catabolism does not require the active site of lipoprotein lipase. Williams et al. (30) showed that the carboxyl–terminal domain of lipoprotein lipase (termed LPLC), which lacks the catalytic site, binds to purified LRP and promotes the binding of VLDL to LRP. Examination of a molecular model of lipoprotein lipase derived from the 3–dimensional structure of pancreatic lipase revealed a hydrophobic loop spanning residues 387–394 that was suspected to represent a lipoprotein binding site. This was confirmed when Williams et al. (30) mutated Trp^{393} and Trp^{394} to alanine. The mutant molecule was unable to bind to lipoproteins, but was still able to bind to heparin and LRP. These results indicate that the carboxyl–terminal domain of lipoprotein lipase may function both in binding lipoproteins and in mediating their interaction with LRP.

Ligand presentation: role of cell surface proteoglycans

A feature of LRP biology emerging from several studies is that additional cell–surface molecules appear capable of facilitating the LRP–mediated uptake of certain ligands. This became apparent in studies examining the lipoprotein lipase–promoted uptake of VLDL (29). During the course of these experiments, it was observed that although anti–LRP IgG blocked the degradation of lipoprotein lipase–enriched VLDL, LRP antibodies had little effect on the cell–surface binding of this ligand. These results indicate that other cell–surface molecules participate in the initial binding of these complexes to the cell. Because lipoprotein lipase is known to bind to proteoglycans, the effect of heparinase–treatment of cells on the lipoprotein lipase–promoted degradation of VLDL particles was examined (29). Incubation of cells with heparinase reduced the surface binding, internalization, and degradation of lipoprotein lipase–enriched VLDL (**Fig. 2**). The catabolism of ^{125}I–labeled α_2M–proteinase complex ($\alpha 2M^*$), which is known to be mediated by LRP, was unaffected by this treatment (Fig. 2). Thus, proteoglycans facilitate the lipoprotein lipase–promoted catabolism of VLDL by cultured cells. Interestingly, proteoglycans are not required for the binding of lipoprotein lipase–enriched VLDL to purified LRP in a cell–free system. A model depicting the role of cell–surface proteoglycans in LRP–mediated catabolism of lipoprotein lipase is summarized in **Fig. 3.** It seems likely that proteoglycans serve to concentrate lipoprotein lipase on the cell surface, thereby enhancing their interaction with LRP. A similar role for proteoglycans in the catabolism of apoE–containing lipoproteins has also been proposed (25). Whether the proteoglycans are internalized along with LRP remains to be determined. The LRP–mediated catabolism of several other molecules has recently been demonstrated to also be facilitated by cell–surface proteoglycans. These ligands include tissue factor pathway inhibitor (31) and thrombospondin (32). This role of surface proteoglycans is somewhat analogous to the presentation of basic fibroblast growth factor with its receptor (33) and to the role of uPAR in presenting uPA for internalization by LRP (see below).

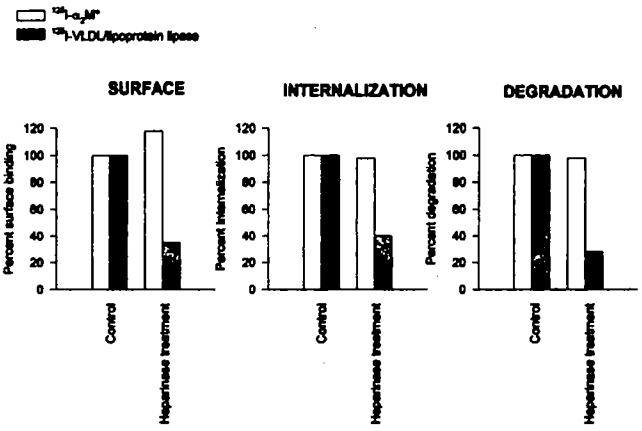

Figure 2. Heparinase treatment of cells inhibits lipoprotein lipase–promoted catabolism of ^{125}I–labeled VLDL. Cells were treated with or without heparinase for 30 min at 37°C. The cells were washed, chilled to 4°C before adding buffer containing 5 nM lipoprotein lipase. After 30 min incubation at 4°C, the cells were incubated with media containing 10 mg/ml ^{125}I–labeled S_f 100–400 particles or 2.8 nM ^{125}I–labeled α_2M^* for 5 h at 37°C. After incubation the cells were washed, and the surface binding, internalization, and degradation determined as described (29).

In vivo role of LRP in lipoprotein catabolism

To investigate the role of LRP in the in vivo catabolism of chylomicron remnants, Mokuno et al. (34) examined the effect of GST–RAP on the uptake and endocytosis of chylomicron remnants by the liver in rats. They found that GST–RAP reduced removal of ^{125}I–labeled chylomicron remnants from the plasma by about 30% and completely abolished endocytosis of these lipoproteins. This observation indicates that a RAP–sensitive receptor, most likely LRP, is playing an important role in the internalization of these particles. RAP also blocked the uptake and endocytosis of ^{125}I–labeled LDL as well, results that are in agreement with the observation that high concentrations of RAP antagonizes ligand uptake by the LDL receptor (21). Using an adenoviral vector to transfer the RAP gene into the liver of normal mice and mice lacking the LDL receptor, Willnow et al. (22) achieved high circulating levels of RAP that completely blocked LRP function. The inactivation of LRP

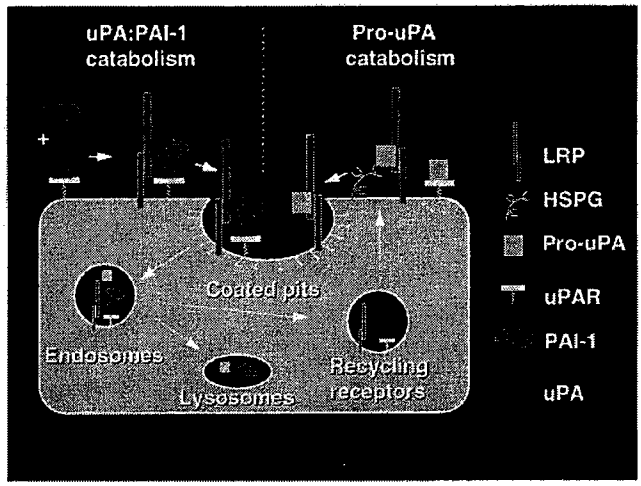

Figure 3. Ligand transfer: model for the role of proteoglycans in facilitating the lipoprotein lipase–mediated catabolism of VLDL. In this model, cell–surface proteoglycans may effectively concentrate ligands, such as lipoprotein lipase, thrombospondin, or tissue factor pathway inhibitor (TFPI) on the cell surface.

was associated with a marked accumulation of chylomicron remnants in the LDL receptor–deficient mice and, to a lesser degree, in normal mice. These studies indicate that the LDL receptor and a RAP–sensitive hepatic receptor, which is most likely LRP, both play an important role in the internalization of chylomicron remnants.

Summary

In vitro experiments have documented the interaction of "activated" lipoproteins with LRP. The activation of the lipoproteins requires addition of exogenous apoE or lipoprotein lipase. Establishing a role for LRP in the in vivo catabolism of lipoproteins has been hampered by the fact that LRP is essential for embryonic development (8), and consequently a genetic deficiency of this receptor has not been identified. In vivo experiments in mice have used RAP as an antagonist to block LRP function (22, 34), and these studies have confirmed that a RAP–sensitive liver receptor, besides the LDL receptor, is also responsible for remnant catabolism. Because LRP and LDL receptor are the only RAP–sensitive receptors currently known to be expressed in the liver, the data support the contention that LRP can function as a remnant receptor.

ROLE OF LRP IN ENDOCYTOSIS OF PROTEINASES AND PROTEINASE–INHIBITOR COMPLEXES

Overview

Tissue–type plasminogen activator (tPA) and urinary–type plasminogen activator (uPA) are multidomain enzymes that catalyze the activation of plasminogen to plasmin. Proteinases such as plasmin are capable of degrading extracellular matrix and of activating latent forms of growth factors. Consequently, proteinases play an important role in normal cellular and tissue function, and inadequate or excessive proteinase activity is likely to be highly detrimental. Thus, mechanisms have been developed to carefully regulate the expression of proteinase activity. In the case of plasminogen, its activation is regulated by the local release of plasminogen activators and inhibitors, the existence of a high–affinity uPA receptor (uPAR) that restricts uPA activity to the cell surface, and the effective removal of tPA and uPA by cell surface receptors. Current evidence suggests that LRP mediates the cellular catabolism of uPA and tPA from sites of expression and from the circulation. Removal of tPA and uPA is an important component in the regulation of profibrinolytic activity, given the potent capability of these enzymes to activate the fibrinolytic pathway. It is also apparent that LRP mediates the lysosomal degradation of inhibited uPA bond to uPAR, and likely acts in concert with uPAR in regulating cell–surface plasmin generation.

Role of LRP in the internalization of pro–uPA and uPA:PAI–1 complexes

An increase in cellular uPA activity has long been associated with angiogenesis, smooth muscle cell migration, and tumor cell metastasis. uPA is synthesized as an inactive single–chain proenzyme, termed pro–uPA, which is converted to the active two–chain molecule by plasmin. Both pro–uPA and uPA bind with high affinity to uPAR (35). uPAR is a three–domain protein that is anchored to the cell surface via a glycosyl–phosphatidylinositol anchor and functions to localize uPA on the cell surface (35). The association of uPA with its receptor, along with the simultaneous binding of plasminogen to the cell surface, provides a potent cell–surface plasmin generating mechanism that has been implicated in several processes requiring proteinase activity, such as cell migration and tissue remodeling (35). Cell–surface uPA activity is regulated by PAI–1, which reacts with uPA to form a complex that is rapidly internalized and degraded. The mechanism by which this occurs was unknown until Nykjær et al.(36) determined that LRP is responsible for this process. They demonstrated that the internalization of ^{125}I–labeled uPA:PAI–1 complexes in monocytes is completely blocked by RAP. Further, they demonstrated that an amino–terminal fragment representing residues 1–135 of uPA (ATF) also blocks the internalization of ^{125}I–labeled uPA:PAI–1 complexes. Because ATF prevents the association of uPA with uPAR, but is not very effective in preventing the binding of uPA:PAI–1 complexes to LRP, it appears that the initial binding of the complex to uPAR facilitates this process. It has been suggested that the efficient removal of inactivated enzymes from the cell surface could be an important mechanism for the regeneration of uPAR from the occupied (uPA:PAI–1) to the unoccupied state (8).

Several features of the catabolism of pro–uPA and uPA:PAI–1 complexes are summarized in **Fig. 4.** uPA:PAI–1 complexes, initially bound to uPAR, are able to

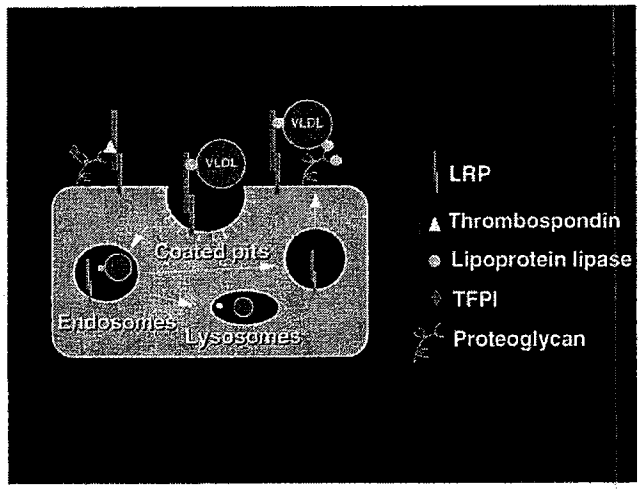

Figure 4. Proposed model for the catabolism of pro-uPA and uPA:PAI-1 complexes. LRP mediates the cellular internalization of both pro-uPA and uPA:PAI-1 complexes. The predominant role of LRP is to mediate the internalization of free pro-uPA or that which may be associated with proteoglycans, whereas pro-uPA associated with uPAR is protected from LRP-mediated catabolism and is localized on the cell surface. It is not until PAI-1 associates with uPAR-bound uPA that the complex is internalized in a process mediated by LRP.

simultaneously bind to LRP (37). The interaction of uPA:PAI–1 complexes with LRP likely involves binding determinants located on PAI–1 and uPA because pro–uPA and PAI–1 are both able to inhibit the binding of ^{125}I–labeled uPA:PAI–1 complexes to LRP in in vitro binding assays (37, 38). To determine whether uPAR is internalized along with LRP, a soluble form of uPAR that lacks the glycosyl–phosphatidylinositol–anchor was used (39). ^{125}I–soluble uPAR in the absence or presence of pro–uPA was not internalized by Chinese hamster ovary cells. However, in the presence of uPA:PAI–1 complexes, significant uptake of ^{125}I–soluble uPAR was detected, which could be blocked by RAP (39). This suggests that uPA:PAI–1 efficiently cross–links uPAR to LRP, which then mediates internalization of the entire complex. This does not occur with pro–uPA alone. Within the endosome, the ligand dissociates from the receptor while LRP is recycled back to the cell surface. At this time, the fate of uPAR is not known, but very likely it also recycles back to the cell surface.

LRP also binds directly to pro–uPA with K_D values that are approximately 15– to 20–fold weaker than that measured for the binding of the uPA:PAI–1 complex to LRP (38). These results suggested that LRP may mediate the cellular catabolism of pro–uPA independent of PAI–1. This was confirmed in cellular uptake experiments (38) that found that the human hepatoma cell line Hep G2 rapidly internalizes and degrades ^{125}I–labeled pro–uPA. Both the internalization and degradation are completely blocked by LRP antibodies and by RAP, indicating that LRP is mediating this process. To investigate the form of uPA internalized, the ligand was recovered from the cells after internalization and evaluated by SDS polyacrylamide gel electrophoresis (38). These experiments revealed that single–chain uPA was internalized and did not require activation to the two–chain molecule or subsequent complex formation with PAI–1 for internalization to occur. Thus, LRP appears capable of mediating the cellular internalization of pro–uPA directly.

The involvement of uPAR in catabolism of pro–uPA has been recently examined. Nykjær et al. (37) found that soluble uPAR blocked the binding of pro–uPA to LRP, suggesting that uPAR may protect pro–uPA from LRP–mediated internalization. The suggestion that uPAR protects pro–uPA from LRP–mediated internalization is an attractive hypothesis, and explains the observation that in certain cell types pro–uPA remains on the cell surface for long periods of time (40). On the other hand, Hep G2 cells (38) and fibroblasts rapidly internalize pro–uPA in a process mediated by LRP. This may result from low levels of uPAR and high levels of LRP within these cells. Also, because the kringle domain of uPA is known to bind to heparin (41), pro–uPA may interact with cell–surface proteoglycans, which could facilitate its transfer to LRP for subsequent internalization and degradation in a mechanism that is somewhat analogous to the participation of cell–surface proteoglycans in the LRP–mediated catabolism of lipoprotein lipase (29). Additional experiments are required to determine whether or not this is the case.

Summarizing all of the available evidence, the predominant role for LRP is to mediate the internalization of free pro–uPA or that which may be associated with proteoglycans, whereas the pro–uPA associated with uPAR is localized on the cell surface and able to mediate cell–surface plasminogen activation. It is not until PAI–1 associates with uPAR–bound uPA that the uPA:PAI– 1 is internalized in a process mediated by LRP. During this latter process, it appears that uPAR is also recycled through the endosomal pathway along with the uPA:PAI–1 complex and is delivered back to the cell surface in an unoccupied state.

Role of LRP in mediating the cellular internalization and degradation of tPA

Significant progress has been made recently in thrombolytic therapy, and tPA has been shown to be an effective thrombolytic agent (42). However, the effectiveness of this agent is hampered somewhat by its rapid clearance from the circulation (42). Three distinct mechanisms have been identified that are responsible for the removal of t–PA from the plasma (43). The first mechanism involves recognition of the mannose–rich oligosaccharide found on the kringle 1 domain of t–PA by the mannose receptor, which is found primarily on endothelial cells. The second mechanism for t–PA clearance occurs after reaction of t–PA with its inhibitor, plasminogen activator inhibitor type 1 (PAI–1). Using ligand blotting experiments, Orth et al. (44) demonstrated that tPA:PAI–1 complexes bind to LRP. Further, they demonstrated that COS–1 cells, a simian fibroblastlike transformed cell line that contains LRP, are able to internalize and degrade these complexes. This process is blocked by

LRP antibodies and by RAP. These data provide compelling evidence that LRP is capable of mediating the internalization of t–PA:PAI–1 complexes, and thus can function as a hepatic receptor responsible for the clearance of tPA:PAI–1 complexes.

A third mechanism for the clearance of tPA involves a PAI–1 independent pathway. In this pathway, an amino–terminal epitope located on the B–loop of the EGF–like domain of t–PA is recognized by an hepatic receptor (43, 45). RAP completely blocks the cellular uptake and degradation of ^{125}I–labeled tPA in cultured fibroblasts (45), which suggests that LRP is responsible for tPA catabolism. This was confirmed by demonstrating that fibroblasts that lack LRP do not internalize ^{125}I–labeled tPA (45). Together, these data suggest that LRP mediates the cellular uptake of tPA, leading to its degradation.

To investigate the role of LRP in mediating the in vivo clearance of tPA in rats, the effect of RAP on the rate of ^{125}I–labeled tPA removal from the circulation was investigated (43). It was observed that infusion of 10 mg of RAP prolonged the plasma half–life of ^{125}I–labeled tPA from 1 min to 5–6 min. These results confirm that a RAP–sensitive hepatic receptor, most likely LRP, is involved in the clearance of t–PA from the circulation.

Summary

LRP plays an important role in regulating the expression of proteinase activity by directly binding and mediating the internalization of uPA and tPA, two potent plasminogen activators. The removal of these two enzymes decreases the profibrinolytic potential of the extracellular environment, leading to a thrombotic state. This may have relevance in certain pathological situations, such as atherosclerotic lesions, where LRP expression in macrophages and smooth muscle cells has been reported (46).

In addition to binding to uPA and tPA, LRP also mediates the internalization of proteinase–inhibitor complexes. The removal of uPA:PAI–1 complexes from uPAR is of special interest, because it has been suggested that receptor–mediated clearance of the complexes may be necessary to regenerate binding sites for pro–uPA that might be necessary for sustained proteolysis (8).

ROLE OF LRP IN THE CATABOLISM OF MATRIX PROTEINS

In addition to its role in proteinase and lipoprotein catabolism, recent data indicate that LRP plays a role in the catabolism of thrombospondin (TSP) (32). Although the function of TSP is not well understood, it appears to be involved in platelet aggregration, smooth muscle cell growth, and angiogenesis. TSP is synthesized by endothelial cells, fibroblasts, and smooth muscle cells, and newly synthesized thrombospondin is either incorporated into the extracellular matrix or binds to the cell surface where it is rapidly internalized and degraded (47). In examining the catabolism of ^{125}I–labeled TSP, Mikhailenko et al. (32) demonstrated that both the cellular internalization and degradation of this molecule are completely inhibited by RAP and by antibodies against LRP, indicating that LRP mediates the cellular internalization of TSP. Like lipoprotein lipase, the efficient catabolism of TSP requires the participation of cell–surface proteoglycans, because digestion of cells with heparitinase markedly reduces the extent of LRP– mediated TSP degradation. Purified TSP binds with high affinity to LRP (K_D of 3–20 nM); like other LRP ligands, its binding is blocked by RAP (32). The ability of LRP to directly bind and mediate the cellular internalization and degradation of TSP indicates that this receptor plays an important role in regulating TSP levels.

The significance of the degradative pathway for TSP is not fully apparent at present. However, TSP appears to have a diverse role in regulating cellular proliferation, adhesion, and migration. For example, TSP has been suggested to directly activate transforming growth factor–β (48), a potent growth regulatory protein normally secreted from cells in a latent form. These and other studies suggest that TSP may affect a variety of biological processes, and if so, it would be important to have a mechanism for rapid catabolism of TSP to regulate its extracellular levels.

POTENTIAL ROLE OF LRP IN PATHOPHYSIOLOGICAL PROCESSES

Atherosclerotic lesions result from an excessive inflammatory response to numerous forms of insult (49). The development of these lesions involve several processes, which include the proliferation of smooth muscle cells and macrophages, the formation of a connective tissue matrix, and the accumulation of smooth muscle cells and macrophages that are engorged with lipid (49). Because LRP mediates the cellular internalization of plasminogen activators, lipoproteins, and thrombospondin, this receptor could play a key role in contributing to events leading to lesion formation. LRP is widely expressed on a variety of cell types, including macrophages, fibroblasts, hepatocytes, smooth muscle cells, syncytiotrophblats, astrocytes, and neurons. Immunocytochemistry and in situ hybridization techniques have detected LRP antigen and message, respectively, in smooth muscle cells and macrophages in both early and advanced lesions (46). Because both apoE and lipoprotein lipase are present in lesions, they could promote the LRP–mediated uptake of cholesterol–rich lipoproteins.

Thus, LRP could play a key role in the development of the lesion by contributing to the formation of foam cells. LRP antigen has also been detected in atherosclerotic lesions in cholesterol–fed normal and Watanabe–heritable hyperlipidemic rabbits (51). Expression of LRP mRNA in aortic tissue increased modestly during cholesterol feeding. This is in marked contrast to the expression of the LDL receptor. Thus, whereas the LDL receptor expression is diminished as cells accumulate cholesterol (9), cholesterol loading of cells has no effect on LRP gene expression due

to the absence of the sterol–dependent response element in the LRP promoter (52).

LRP also regulates the expression of plasmin activity by directly binding and mediating the cellular internalization of urokinase– and tissue–type plasminogen activators. The cellular removal of these two enzymes decrease the local profibrinolytic potential, possibly leading to a thrombotic state at lesion sites. LRP has also recently been shown to regulate the extracellular accumulation of thrombospondin by mediating its cellular uptake and degradation (32). Finally, binding of activated α2M to LRP markedly enhances the TGF–β–induced growth response of cultured rat aortic smooth muscle cells (50). It is apparent that LRP is a multiligand receptor that mediates the catabolism of a number of molecules known to be important in vascular biology. LRP therefore, plays a unique role as an endocytotic receptor and may contribute to some of the processes that lead to the development of lesions. FJ

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Example 18: <u>Process claim where the novelty is in the method steps.</u>

Specification: The specification teaches a method for producing proteins using mitochondria from the fungus *Neurospora crassa*. In the method, mitochondria are isolated from this fungus and transformed with a mitochondrial expression vector which comprises a nucleic acid encoding a protein of interest. The protein is subsequently expressed, the mitochondria is lysed, and the protein is isolated. The specification exemplifies the expression of ∃-galactosidase using the claimed method using a cytochrome oxidase promoter.

Claim:

1. A method of producing a protein of interest comprising;

obtaining *Neurospora crassa* mitochondria,

transforming said mitochondria with a expression vector comprising a nucleic acid that encodes said protein of interest,

expressing said protein in said mitochondria, and

recovering said protein of interest.

Analysis:

A review of the specification reveals that *Neurospora crassa* mitochondrial gene expression is essential to the function/operation of the claimed invention. A particular nucleic acid is not essential to the claimed invention.

A search of the prior art reveals that the claimed method of expression in *Neurospora crassa* is novel and unobvious.

The claim is drawn to a genus, i.e., any of a variety of methods that can be used for expressing protein in the mitochondria.

There is actual reduction to practice of a single embodiment, i.e., the expression of ∃-galactosidase.

The art indicates that there is no substantial variation within the genus because there are a limited number of ways to practice the process steps of the claimed invention.

The single embodiment is representative of the genus based on the disclosure of *Neurospora crassa* mitochondria as a gene expression system, considered along with the level of skill and knowledge in the gene expression art. One of skill in the art would recognize that applicant was in possession of all of the various expression methods necessary to practice the claimed invention.

Conclusion:

The claimed invention is adequately described.

Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 974–978, February 1996
Immunology

Improved antitumor activity of a recombinant anti-Lewis^y immunotoxin not requiring proteolytic activation

(cancer therapy/*Pseudomonas* exotoxin/monoclonal antibody B1 Fv fragment/disulfide-stabilized Fv fragment/protein engineering)

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Contributed by Ira Pastan, October 16, 1995

ABSTRACT **B1(dsFv)-PE33 is a recombinant immunotoxin composed of a mutant form of *Pseudomonas* exotoxin (PE) that does not need proteolytic activation and a disulfide-stabilized Fv fragment of the anti-Lewis^y monoclonal antibody B1, which recognizes a carbohydrate epitope on human carcinoma cells. In this molecule, amino acids 1–279 of PE are deleted and domain Ib (amino acids 365–394) is replaced by the heavy chain variable region (V_H) domain of monoclonal antibody B1. The light chain (V_L) domain is connected to the V_H domain by a disulfide bond. This recombinant toxin, termed B1(dsFv)-PE33, does not require proteolytic activation and it is smaller than other immunotoxins directed at Lewis^y, all of which require proteolytic activation. Furthermore, it is more cytotoxic to antigen-positive cell lines. B1(dsFv)-PE38 has the highest antitumor activity of anti-Lewis^y immunotoxins previously constructed. B1(dsFv)-PE33 caused complete regression of tumors when given at 12 μg/kg (200 pmol/kg) every other day for three doses, whereas B1(dsFv)-PE38 did not cause regressions at 13 μg/kg (200 pmol/kg). By bypassing the need for proteolytic activation and decreasing molecular size we have enlarged the therapeutic window for the treatment of human cancers growing in mice, so that complete remissions are observed at 2.5% of the LD_{50}.**

Recombinant Fv-immunotoxins are chimeric proteins in which a truncated toxin is fused to an Fv fragment of an antibody. The Fv region targets antigens on tumor cells and the toxin moiety kills the cell. Fv-immunotoxins have very good cytotoxic activity on human tumor cell lines and can cause complete regression of established human tumor xenografts in mice (1–3). Several Fv-immunotoxins are currently being evaluated in clinical or preclinical trials (4). Originally, the Fv fragments of the recombinant toxins were designed in a single-chain form (scFv-immunotoxins), in which the heavy and light chain variable region (V_H and V_L) domains are connected by a flexible peptide linker (5, 6). Subsequently a method was developed to stabilize the Fv fragments by an interchain disulfide bond that connects structurally conserved framework regions of the V_H and V_L domains (refs. 7–9 and reviewed by Reiter and Pastan in ref. 10). Such disulfide-stabilized dsFv-immunotoxins are much more stable than scFv-immunotoxins, and some have improved antigen-binding affinities and improved antitumor activities (11). A major advantage of using Fv fragments, which are the smallest functional modules of antibodies, in recombinant immunotoxins is that these molecules are significantly smaller than chemical conjugates made with whole antibodies. This allows them to effectively penetrate into solid tumors (12, 13).

Pseudomonas exotoxin (PE)-based recombinant immunotoxins require proteolytic activation. Domain II of the toxin is cleaved between amino acids 279 and 280, a reaction that is catalyzed by the enzyme furin (14, 15). This step could be rate limiting, because the furin concentration in cells is low and some cancer cells may be furin deficient. Therefore, we have constructed a recombinant immunotoxin that does not need proteolytic activation. Furin cleavage generates a 37-kDa carboxyl-terminal fragment of PE, amino acids 280–613 (PE37), that contains the translocating and ADP-ribosylation activity of PE (16, 17). If a functional Fv fragment could be inserted into PE37 without destroying its ADP-ribosylation activity or translocating ability and the Fv still retained its binding affinity, then the recombinant molecule should be more active than a toxin which needs to be proteolytically processed.

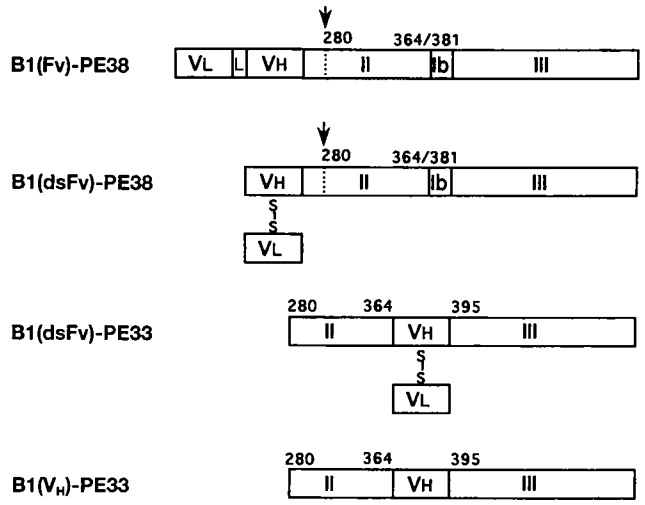

FIG. 1. Schematic of expressed proteins. Positions of amino acids that span PE sequences are numbered. The arrow marks the proteolytic site of PE for activation. S—S shows the disulfide bond linkage between the Fv fragments. L, peptide linker; II, PE domain II for translocation; Ib, PE domain Ib (function unknown); III, PE domain III for ADP-ribosylation of EF2.

Monoclonal antibody (mAb) B1 is a murine antibody directed against Lewis^y-related carbohydrate antigens, which are abundant on the surface of many carcinomas (18). mAb B1 has been used to make both single-chain and disulfide-stabilized Fv-immunotoxins (18–20). These agents are capable of causing complete regression of established xenografts in nude mice (20). To develop a recombinant immunotoxin that is small and stable and does not need proteolytic processing, we have replaced domain Ib (amino acids 365–394) of PE37 with the

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Abbreviations: mAb, monoclonal antibody; V_H and V_L, variable heavy and light chain, respectively; scFv and dsFv, single-chain and disulfide-stabilized Fv, respectively; PE, *Pseudomonas exotoxin*; Mes, 4-morpholinecthanesulfonic acid.

*To whom reprint requests should be addressed.

V_H fragment of mAb B1 and linked the V_H domain to the V_L domain with a disulfide bond (Fig. 1). We find that the resulting molecule, B1(dsFv)-PE33, is more active than any previous mAb B1-containing immunotoxin.

MATERIALS AND METHODS

Construction of Plasmids for Expression of B1(dsFv)-PE33. "Sticky feet"-directed mutagenesis (21) using uracil-containing pDF1 as a template was used to construct the expression plasmid encoding for B1(V_H)R44C-PE33, the component of the intramolecularly inserted dsFv-immunotoxin. The construction of plasmids pDF1, which encodes PE37, which starts at methionine followed by PE amino acids 281–613 (a truncated form of PE that does not require proteolytic activation), and pB1V_HR44C-PE38, which encodes the single-domain B1(V_H)R44C-PE38 immunotoxin, has been described (16, 20). The B1(V_H)R44C DNA was PCR amplified by using the plasmid pB1V_HR44C-PE38 as a template and oligo primers CT119 and 5′-phosphorylated CT120. The forward PCR primer CT119, 5′-GGCAACGACGAGGCCGGCGCGGCCAACGGCGGTGGCGGATCCGAGGTGCAGCTGGTGGAATCTGGA-3′, had sequences that are identical to the DNA encoding PE residues 356–364 and a peptide linker GGGGS inserted at the 5′ end of V_H, and a *Bam*HI site was created (underlined). The reverse PCR oligonucleotide primer CT120, 5′-GTCGCCGAGGAACTCCGCGCCAGTGGGCTCGGGACCTCCGGAAGCTTTTGC-3′, had sequences that are complementary to the DNA encoding PE residues 395–403 and an Fv-toxin junction (connector) inserted at the 3′ end of V_H, and a *Hin*dIII site was created (underlined). The PCR product was purified and annealed with a uracil-containing single-stranded DNA prepared by the rescue of pDF1 phagemid with an M13K07 helper phage (Bio-Rad). The DNA was extended and ligated according to the Muta-Gene mutagenesis kit (Bio-Rad). Because the annealing efficiency of the PCR fragment to the single-stranded template and the mutagenesis efficiency were low (≈10%), the DNA pool of the mutagenesis reaction was digested with a restriction endonuclease which cuts a unique site in the domain Ib region but not in B1(V_H). This extra digestion step improved the mutagenesis efficiency to more than 50%. Correct clones were identified by DNA restriction analysis and verified by DNA sequencing. The resulting immunotoxin clone was named pB1(V_H)R44C-PE33 or pCTK104. It encodes a single-domain B1(V_H)-immunotoxin in which the V_H domain replaces the domain Ib region (amino acids 365–394) of PE37. The plasmid pB1V_LA105CSTOP encodes B1(V_L)A105C, which is a component of dsFv-immunotoxin as described previously (20).

Production of Recombinant Immunotoxin. The components of the disulfide-stabilized immunotoxins B1(V_H)R44C-PE38, B1(V_H)R44C-PE33, and B1(V_L)A105C or the single-chain immunotoxin B1(Fv)-PE38 were produced in separate *Escherichia coli* BL21(λDE3) (22) cultures harboring the corresponding expression plasmid. All recombinant proteins accumulated in inclusion bodies. Disulfide-stabilized immunotoxins were obtained by mixing equimolar amounts of solubilized and reduced inclusion bodies essentially as described (23), except that the final oxidation step was omitted and refolding was carried out at pH 9.5. Properly folded disulfide-stabilized and single-chain immunotoxins were purified by sequential ion-exchange (Q-Sepharose and Mono Q) followed by size-exclusion chromatography on a TSK G3000SW (TosoHaas) column as described (7).

Analysis of Immunotoxins. The cytotoxic activity of immunotoxins was determined by inhibition of protein synthesis as described (24). For competition assays designed to prove the specificity of the recombinant immunotoxins, we changed the medium and added 50 μg of antibody per well 30 min prior to the addition of the immunotoxin. Thermal stability of the immunotoxins was determined by incubating them at 100 μg/ml in phosphate-buffered saline (PBS; 6.7 mM sodium phosphate, pH 7.4/150 mM NaCl) at 37°C for 8 h, followed by analytical chromatography on a TSK G3000SW (TosoHaas) column to separate the monomers from larger aggregates (8). Relative binding affinities of the immunotoxins were determined by adding ^{125}I-labeled B1-IgG to 10^5 A431 cells as a tracer with various concentrations of the competitor. The binding assays were performed at 4°C for 2 h in RPMI medium 1640 containing 1% bovine serum albumin and 50 mM Mes (Sigma) as described (25).

Toxicity and Antitumor Activity in Nude Mice. The single-dose mouse LD_{50} was determined by using female BALB/c mice (6–8 weeks old, ≈20 g), which were given a single i.v. injection of different doses of B1(dsFv)PE38 or B1(dsFv)PE33 diluted in 200 μl of PBS containing 0.2% human serum albumin (PBS-HSA). Mice were followed for 2 weeks after injection. Athymic (*nu/nu*) mice, females 6–8 weeks old, ≈20 g, were injected s.c. on day 0 with 3×10^6 A431 cells suspended in RPMI medium without fetal bovine serum. By day 5, tumors were about 50–70 mm^3 in size. Mice were treated on days 5, 7, and 9 by i.v. injections of different doses of immunotoxins diluted in PBS-HSA. Tumors were measured with a caliper and the tumor volumes were calculated by using the formula volume = length × width^2 × 0.4.

RESULTS

Plasmid Constructions and Production of B1(dsFv)-PE33. Our goal was to construct an active recombinant immunotoxin that was smaller than the current generation of recombinant immunotoxins and that did not need intracellular proteolytic cleavage for activation. To do this we inserted the B1 dsFv fragment between domains II and III by replacing domain Ib of PE37, a truncated form of PE that contains only the portion of the toxin that undergoes translocation to the cytosol. As shown in Fig. 1, the V_H domain replaces amino acids 365–394 of PE37 and the V_L domain is connected to the V_H domain by a disulfide bond engineered into the framework region, with cysteines introduced at position 44 of V_H and position 105 of V_L (7). The resulting recombinant immunotoxin, termed B1(dsFv)-PE33, is 5 kDa smaller than B1(dsFv)-PE38 or B1(Fv)-PE38 (Fig. 1). In the toxin portion, Cys-287 was changed to a Ser to reduce the chance of incorrect disulfide bond formation (26). B1(V_H)R44C is inserted after amino acid 364 of PE and the insert is preceded by a small flexible peptide linker, GGGGS. Following the V_H domain is another peptide, KASGGPE, C3 connector (27), that connects the carboxyl terminus of V_H to amino acid 395 of PE.

The "sticky feet"-directed mutagenesis protocol used for the construction of B1(V_H)R44C-PE33 is described in *Materials and Methods*. Immunotoxins were expressed in *E. coli* BL21(λDE3); cultures for expressing the components of the dsFv-immunotoxin were prepared separately. The immunotoxins were purified by refolding of inclusion bodies in a redox-shuffling buffer and sequential ion-exchange and gel-filtration chromatography as described in *Materials and Methods*. The proteins obtained were more than 95% homogeneous and had the expected molecular mass of 59 kDa on SDS/PAGE as shown in lane 2 of Fig. 2. In the presence of the reducing agent 2-mercaptoethanol, the dsFv-immunotoxin, B1(dsFv)-PE33, dissociated into its two components (lane 4), B1(V_L) and B1(V_H)-PE33. The apparent molecular masses of these components are 13 kDa and 46 kDa, respectively. We also produced the single-domain B1(V_H)-PE33 immunotoxin as shown in Fig. 2. The yield of either B1(dsFv)-PE33 or B1(V_H)-PE33 was 8–10% of the total protein present in inclusion bodies.

Improved Cytotoxic Activity of B1(dsFv)-PE33 Toward B1-Antigen-Expressing Cell Lines. The cytotoxicity of B1(dsFv)-

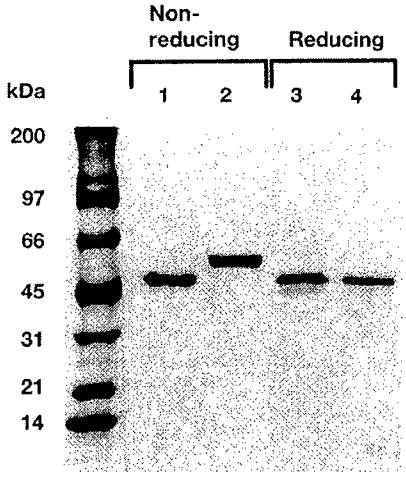

FIG. 2. Purity of B1(dsFv)-PE33 and B1(V_H)-PE33: SDS/4–20% PAGE. Lanes: 1, B1(V_H)-PE33, nonreduced; 2, B1(dsFv)-PE33, nonreduced; 3, B1(V_H)-PE33, reduced; and 4, B1(dsFv)-PE33, reduced. The left lane contains mass markers.

PE33 was determined by measuring the decrease in incorporation of [^{3}H]leucine by various human cancer cell lines after treatment with immunotoxin (24). B1(dsFv)-PE38 and B1(V_H)-PE33 (no light chain) were included for comparison. Fig. 3*A* and Table 1 show that all three proteins are cytotoxic to cells expressing B1 antigen (A431, MCF7, CRL1739, and LNCaP) but not to cells that do not bind mAb B1 (L929 and HUT102). In this assay, B1(dsFv)-PE33 had an IC_{50} of 0.25 ng/ml on A431 cells and 0.35 ng/ml on MCF7 cells. We found

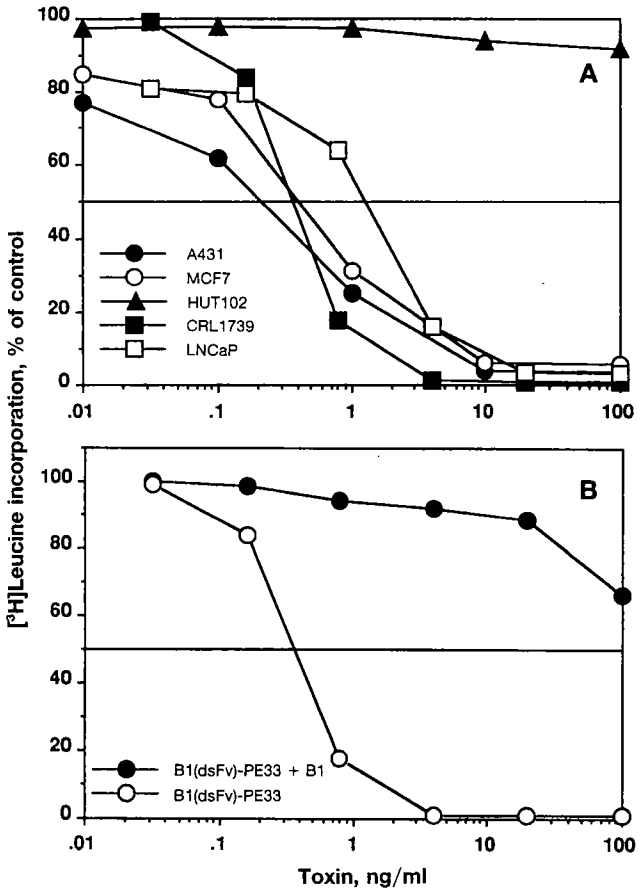

FIG. 3. (*A*) Toxicity of B1(dsFv)-PE33 for various cell lines. (*B*) mAb B1 inhibition of the cytotoxicity of B1(dsFv)-PE33 for A431 cells.

Table 1. Cytotoxicity of B1 immunotoxins toward various cell lines

Cell line*	Cancer type	Antigen expression†	IC_{50},‡ ng/ml B1(dsFv)-PE38	B1(dsFv)-PE33	B1(V_H)-PE33
A431	Epidermoid	+++	0.5	0.25	2.0
MCF7	Breast carcinoma	+++	0.9	0.35	4.0
CRL1739	Gastric	+++	0.4	0.31	ND
LNCaP	Prostate	+	7.0	1.3	ND
HUT102	T-cell leukemia	−	>1000	>1000	>1000
L929	Mouse fibroblast	−	>1000	>1000	>1000

ND, not determined.
*All the cell lines except L929 are of human origin.
†The level of antigen expression is marked +++, +, and − for strong, medium, and no detectable expression in immunofluorescence, respectively.
‡Cytotoxicity data are given as IC_{50} value, the concentration of immunotoxin that causes a 50% inhibition of protein synthesis after a 20-h incubation with immunotoxin.

that B1(dsFv)-PE33 was more active on all antigen-positive cell lines compared with B1(dsFv)-PE38, which requires proteolytic processing. To analyze whether the cytotoxicity of B1(dsFv)-PE33 was specific, competition experiments were carried out with an excess of mAb B1. Fig. 3*B* shows that the intoxication of A431 carcinoma cells by B1(dsFv)-PE33 is due to the specific binding to the B1 antigen, since its cytotoxicity was blocked by excess mAb B1. We also tested B1(V_H)-PE33, which is not associated with light chain, and found that it was only about 10-fold less cytotoxic (IC_{50} of 2 ng/ml on A431 cells) than B1(dsFv)-PE33 (Table 1), indicating the heavy chain has a major role in antigen binding. However, a related single-domain immunotoxin, B3(V_H)-PE38, which requires proteolytic processing for activation, is much less active, with an IC_{50} of 40 ng/ml on A431 cells (28).

Antigen Binding of B1(dsFv)-PE33. To determine whether the improved cytotoxicity of B1(dsFv)-PE33 is due to improved binding, we analyzed its binding affinity to antigen-positive cells (A431 cells) by competition assays, in which increasing concentrations of each immunotoxin competed for the binding of ^{125}I-labeled B1 IgG to A431 cells at 4°C. The results shown in Fig. 4 indicate that B1 IgG, B1(dsFv)-PE38, B1(dsFv)-PE33, and B1(V_H)-PE33 competed for the binding of ^{125}I-labeled B1 IgG to A431 cells by 50% at 40 nM, 2 μM, 3.5 μM, and 25 μM, respectively. Thus, the binding affinity of

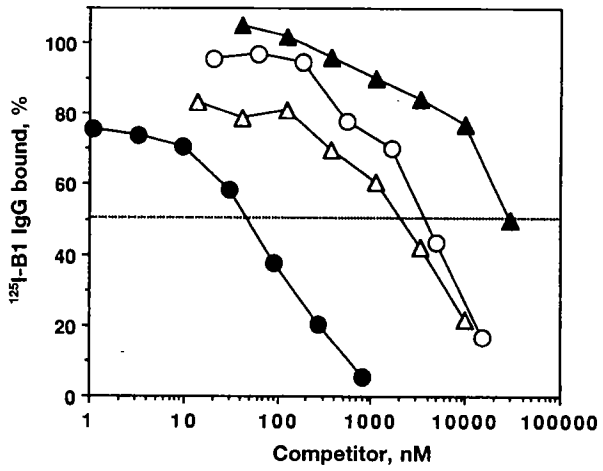

FIG. 4. Binding of B1(dsFv)-PE33, B1(dsFv)-PE38, and B1(V_H)-PE33 to A431 cells: Competitive binding analysis of the ability of purified B1(dsFv)-PE33, B1(dsFv)-PE38, and B1(V_H)-PE33 to inhibit the binding of ^{125}I-labeled B1 IgG to A431 cells overexpressing B1 antigen. ●, B1 IgG; ○, B1(dsFv)-PE33; △, B1(dsFv)-PE38; and ▲, B1(V_H)-PE33.

Table 2. Thermal stability of immunotoxins

Immunotoxin	Amount, % of control	
	Monomer	Aggregates
B1(dsFv)-PE38	100	<0.1
B1(Fv)-PE38	≈40	≈60
B1(dsFv)-PE33	100	<0.1

The thermal stability of immunotoxins to heat treatment was determined by incubation at 0.1 mg/ml in PBS at 37°C for 8 h, followed by analytical chromatography on a TSK G3000SW (TosoHaas) column, to quantitate the amount of monomers and aggregates compared to the untreated immunotoxins.

B1(dsFv)-PE33 is slightly reduced compared with B1(dsFv)-PE38. Therefore, the improved cytotoxicity cannot be due to improved binding, suggesting that elimination of the requirement for proteolytic activation is probably responsible for the improved cytotoxicity. The single-domain immunotoxin B1(V_H)-PE33 exhibited a 10-fold lower binding affinity relative to the dsFv-immunotoxins, which is consistent with its diminished cytotoxicity (Table 1).

Stability of Immunotoxin. The stability of immunotoxins at 37°C is an important factor in their usefulness as therapeutic agents. The stability of an immunotoxin is governed by its tendency to aggregate at 37°C. The thermal stability of B1(dsFv)-PE33, B1(dsFv)-PE38, and B1(Fv)-PE38 was determined by measuring the amount of aggregation and inactivation at 37°C as described in *Materials and Methods.* We found that both B1(dsFv)-PE33 and B1(dsFv)-PE38 were monomers before incubation in PBS at 37°C and remained monomeric for 8 h (Table 2). In contrast, the single-chain immunotoxin B1(Fv)-PE38 formed >60% aggregates after an 8-h incubation at 37°C (Table 2; also see ref. 20). Following the 8-h incubation at 37°C, B1(dsFv)-PE33 and B1(dsFv)-PE38 retained almost all their initial cytotoxic activity, while B1(Fv)-PE38 lost 75% of its initial cytotoxic activity (20). Thus, both B1(dsFv)-PE38 and B1(dsFv)-PE33 are extremely stable at 37°C, presumably because they do not tend to aggregate as do the scFv-immunotoxins.

Toxicity of Immunotoxins in Mice. The toxicity of single doses of the immunotoxins B1(dsFv)-PE33 and B1(dsFv)-PE38 was measured by i.v. injections of different amounts of immunotoxin into BALB/c mice. The mice were observed for 14 days after injection. The LD_{50} of both immunotoxins was found to be 0.5 mg/kg, similar to the LD_{50} determined for the B1(dsFv)-PE38 as well as other anti-Lewis^y Fv-immunotoxins (23). The results show that even though the immunotoxin is more active on target cells because it does not require proteolytic activation, it is not more toxic to mice. This toxicity in mice is presumed to be due to nonspecific uptake of the toxin moiety by the liver (29).

Improved Antitumor Activity of B1(dsFv)-PE33. To determine whether the improved cytotoxicity *in vitro* is accompanied by an increase in antitumor activity, B1(dsFv)-PE33 and B1(dsFv)-PE38 were compared by assessing their ability to cause regression of established human carcinoma xenografts in nude mice. Nude mice were injected with 3×10^6 A431 cells s.c. on day 0. Beginning 5 days later, when tumors averaged

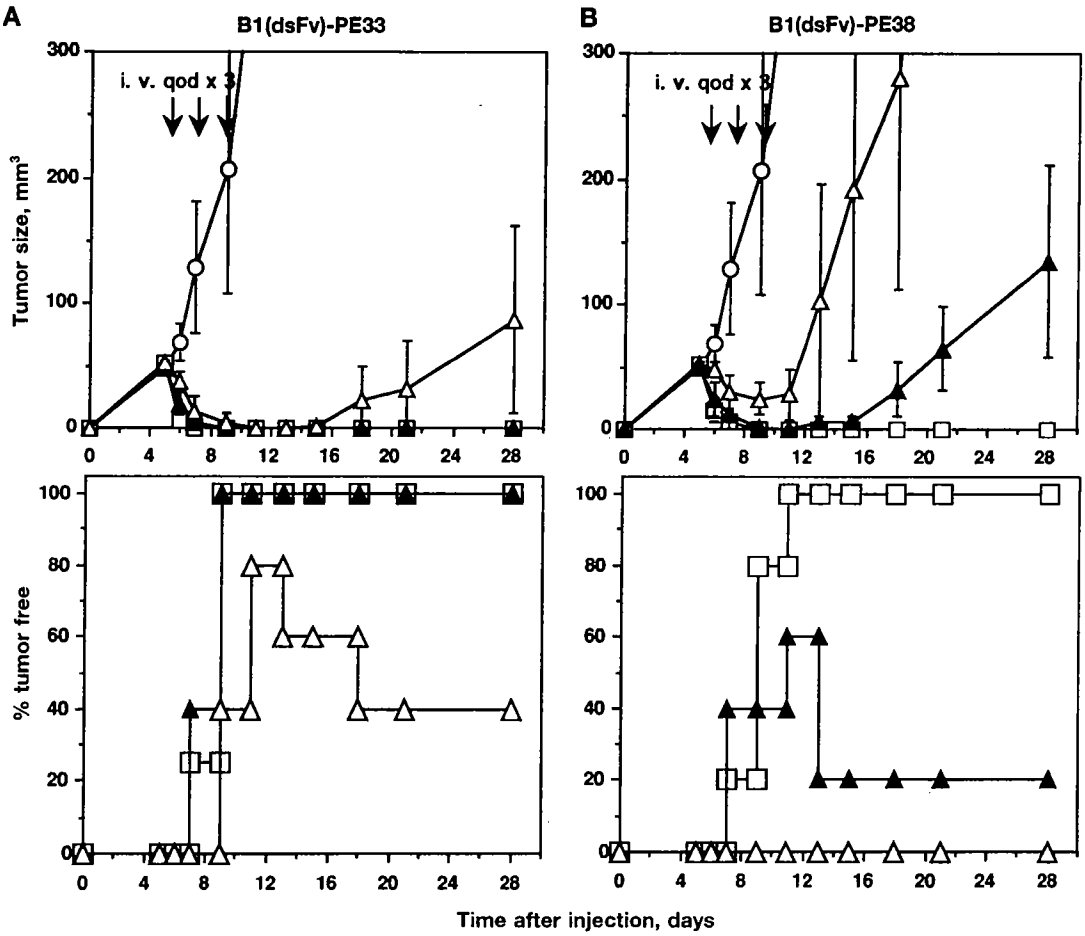

FIG. 5. Antitumor effect and durability of complete remissions due to B1(dsFv)-PE33 and B1(dsFv)-PE38 in a nude mouse model. Groups of five mice were injected s.c. with 3×10^6 A431 cells on day 0 and were treated by i.v. injections of B1(dsFv)-PE33 (*Left*) or B1(dsFv)-PE38 (*Right*) on days 5, 7, and 9 (indicated by arrows), when the tumors were established. Control mice were treated with PBS-HSA. Error bars represent the standard error of the data. ○, Control; □, 400 pmol/kg; ▲, 200 pmol/kg; and △, 100 pmol/kg.

50–70 mm^3 in volume, the mice were treated with i.v. injections on days 5, 7, and 9 of various doses of immunotoxin. Control mice were treated with PBS-HSA only. As shown in Fig. 5, both immunotoxins demonstrated significant dose-dependent antitumor activity. B1(dsFv)-PE38 caused only partial regression of A431 tumors at the 6.5 μg/kg (100 pmol/kg) dose level, whereas B1(dsFv)-PE33 at the same dose caused complete disappearance of the tumors (Fig. 5). Furthermore, the tumors treated with B1(dsFv)-PE38 at 200 pmol/kg (13 μg/kg) regressed completely after the third injection but regrew within a few days, whereas B1(dsFv)-PE33 at 200 pmol/kg (12 μg/kg) caused complete regressions that lasted over 1 month in 5 of 5 animals. These results indicate that B1(dsFv)-PE33 has significantly better antitumor activity than B1(dsFv)-PE38. Thus, the improved cytotoxicity *in vitro* results in improved antitumor activity in animals.

DISCUSSION

We have developed a recombinant immunotoxin composed of a dsFv fragment of mAb B1 and a truncated form of PE that is smaller than other recombinant PE-derived immunotoxins and does not require intracellular proteolytic activation. Another advantage is that it is more active *in vitro* and a more potent antitumor agent than immunotoxins made with the same antibody that require proteolytic processing (20).

Location for B1(dsFv) Insertion in PE33. The B1 dsFv fragment was inserted between the translocation domain and ADP-ribosylation domain of PE, replacing domain Ib. The rationale for this design is that domain Ib (amino acids 364–395) is not essential for the cytotoxic activity (30), and it can be completely deleted from immunotoxins without loss of activity. In fact, it is also possible to delete a portion of domain II (amino acids 343–364) without loss of activity. In addition, analysis of the proposed structure of B1(dsFv)-PE33 by using computer graphics (N. Kurochkina, C.-T.K. and I.P., unpublished results) indicates that domain Ib should be a good location for insertion of the dsFv fragment, because the complementarity-determining regions of the Fv should be "free" to interact with antigen. The results in Fig. 4 show that the presence of B1(dsFv) in this region only minimally affected antigen binding. In another study, we have inserted a dsFv fragment of mAb e23, which binds to the erbB2 antigen, near the carboxyl terminus of PE35 at amino acid 607; this location was found to significantly decrease antigen binding of e23(dsFv) to its antigen (31). It is necessary to investigate whether the domain Ib location is useful for the insertion of other dsFvs.

Improved Antitumor Activity of B1(dsFv)-PE33. To compare the antitumor activity of B1(dsFv)-PE33 with B1(dsFv)-PE38, we used the A431 human epidermoid carcinoma model to evaluate the ability of both immunotoxins to cause complete regression of tumors. B1(dsFv)-PE38 is very potent in antitumor activity (20). We found that when the nude mice were treated with three doses of 200 pmol/kg, given every other day, B1(dsFv)-PE38 caused significant tumor regressions but did not produce cures. In contrast, B1(dsFv)-PE33 caused complete remissions and cures in all animals at the same dose of 200 pmol/kg (Fig. 5). Thus, B1(dsFv)-PE33 has better antitumor activity than B1(dsFv)-PE38. Since both B1(dsFv)-PE33 and B1(dsFv)-PE38 have the same toxicity in mice, the PE33 immunotoxin has a larger therapeutic window. The effective dose causing complete remissions in nude mice is 2.5% of the LD_{50}. This makes B1(dsFv)-PE33 a good candidate for clinical development. The improved antitumor activity of B1(dsFv)-PE33 over B1(dsFv)-PE38 is a consequence of better cytotoxicity *in vitro* due to lack of requirement for proteolytic activation and possibly smaller size for better tumor penetration. Since the efficiency of proteolytic activation can vary in different types of cells, the type of recombinant immunotoxin described here may be more useful than the previous generation of molecules, which require proteolytic activation.

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Helmbrook

Medline

Transforming growth factor alpha-Pseudomonas exotoxin fusion protein prolongs survival of nude mice bearing tumor xenografts.

Helmbrook DC, Stirdivant SM, Ahern JD, Balishin NL, Patrick DR, Edwards GM, Defeo-Jones D, FitzGerald DJ, Pastan I, Oliff A

Proc Natl Acad Sci U S A 1990 Jun 87:4697-701

BROWSE : Proc Natl Acad Sci U S A • Volume 87 • Issue 12

VIEW : **MEDLINE**, full MEDLINE, related records

Abstract

Transforming growth factor alpha (TGF alpha)-Pseudomonas exotoxin 40 (PE40) is a chimeric protein consisting of an N-terminal TGF alpha domain fused to a C-terminal 40-kDa segment of the Pseudomonas exotoxin A protein. TGF alpha-PE40 exhibits the receptor-binding activity of TGF alpha and the cell-killing activity of PE40. These properties make TGF alpha-PE40 an effective cytotoxic agent for cells that possess epidermal growth factor receptors (EGFR). However, the utility of this protein as an anticancer agent has been unclear because many normal tissues express EGFR and may be damaged by exposure to TGF alpha-PE40. To address this issue, we injected nude mice with a lethal inoculum of either A431 or HT29 human tumor cells that possess EGFR or with Chinese hamster ovary (CHO) tumor cells that lack EGFR. Animals were treated with a derivative of TGF alpha-PE40 in which the cysteine residues are replaced by alanine, termed "TGF alpha-PE40 delta cys," or with saline once a day for 5 days. Mice bearing EGFR+ tumor cells lived significantly ($P < 0.001$) longer when treated with TGF alpha-PE40 delta cys compared with saline-treated controls (median survival: A431 cells, 51.5 vs. 25.5 days; HT29 cells, 101 vs. 47.5 days). TGF alpha-PE40 delta cys did not prolong the survival of mice bearing tumor cells that lack EGFR (median survival: CHO cells, 15.5 vs. 19.5 days). The only toxicity to normal tissues was mild periportal hepatic necrosis. These studies indicate that a therapeutic window exists in vivo for the use of some growth factor-toxin fusion proteins as anticancer agents.

MeSH

Alanine; Animal; Antineoplastic Agents; Cell Line; Cloning, Molecular; Cysteine; Exotoxins; Female; Human; Mice; Mice, Nude; Mutation; Neoplasm Transplantation; Neoplasms; Receptor, Epidermal Growth Factor; Transforming Growth Factors

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Order Document

Beraud

Medline

Immunospecific suppression of encephalitogenic-activated T lymphocytes by chimeric cytotoxin IL-2-PE40.

Beraud E, Lorberboum-Galski H, Chan CC, FitzGerald D, Pastan I, Nussenblatt RB

Cell Immunol 1991 Apr 133:379-89

BROWSE : Cell Immunol • Volume 133 • Issue 2

VIEW : **MEDLINE**, full MEDLINE, related records

Abstract

We examined the action of a chimeric protein, IL-2-PE40, on the development of a T cell-mediated disease of the central nervous system with numerous similarities to multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). EAE is caused by IL-2 receptor-bearing T cells specific for myelin basic protein (BP). We report here that the treatment of Lewis rats with IL-2-PE40 delayed and shortened the course of EAE induced by BP in adjuvant and dramatically prevented EAE mediated by anti-myelin basic protein T line cells. The absence of paralytic signs, the absence of cell infiltration in the central nervous system, and the abatement of cellular immunity to myelin basic protein in the treated rats are direct consequences of the specific mechanism of action of IL-2-PE40. Our data support the notion that IL-2-PE40 may be efficient as an immunosuppressive agent for those disorders in which activated T cells play a crucial role.

MeSH

Animal; Cell Line; Encephalomyelitis, Experimental Autoimmune; Exotoxins; Female; Hypersensitivity, Delayed; Interleukin-2; Myelin Basic Proteins; Pseudomonas aeruginosa; Rats; Rats, Inbred Lew; Receptors, Interleukin-2; Recombinant Fusion Proteins; T-Lymphocytes

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Batra

Medline

Single-chain immunotoxins directed at the human transferrin receptor containing Pseudomonas exotoxin A or diphtheria toxin: anti-TFR(Fv)-PE40 and DT388-anti-TFR(Fv).

Batra JK, Fitzgerald DJ, Chaudhary VK, Pastan I

Mol Cell Biol 1991 Apr 11:2200-5

BROWSE : Mol Cell Biol • Volume 11 • Issue 4

VIEW : **MEDLINE**, full MEDLINE, related records

Abstract

Two single-chain immunotoxins directed at the human transferrin receptor have been constructed by using polymerase chain reaction-based methods. Anti-TFR(Fv)-PE40 is encoded by a gene fusion between the DNA sequence encoding the antigen-binding portion (Fv) of a monoclonal antibody directed at the human transferrin receptor and that encoding a 40,000-molecular-weight fragment of Pseudomonas exotoxin (PE40). The other fusion protein, DT388-anti-TFR(Fv), is encoded by a gene fusion between the DNA encoding a truncated form of diphtheria toxin and that encoding the antigen-binding portion of antibody to human transferrin receptor. These gene fusions were expressed in Escherichia coli, and fusion proteins were purified by conventional chromatography techniques to near homogeneity. In anti-TFR(Fv)-PE40, the antigen-binding portion is placed at the amino terminus of the toxin, while in DT388-anti-TFR(Fv), it is at the carboxyl end of the toxin. Both these single-chain immunotoxins kill cells bearing the human transferrin receptors. However, anti-TFR(Fv)-PE40 was usually more active than DT388-anti-TFR (Fv), and in some cases it was several-hundred-fold more active. Anti-TFR(Fv)-PE40 was also more active on cell lines than a conjugate made by chemically coupling the native antibody to PE40, and in some cases it was more than 100-fold more active.

MeSH

Amino Acid Sequence; Animal; Base Sequence; Binding, Competitive; Cell Survival; Cloning, Molecular; Diphtheria Toxin; Electrophoresis, Polyacrylamide Gel; Escherichia coli; Exotoxins; Human; Immunotoxins; Mice; Molecular Sequence Data; Plasmids; Polymerase Chain Reaction; Receptors, Transferrin; Recombinant Fusion Proteins; Tumor Cells, Cultured

Author Address

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Order Document

Pai

Medline

Antitumor activity of a transforming growth factor alpha-Pseudomonas exotoxin fusion protein (TGF-alpha-PE40).

Pai LH, Gallo MG, FitzGerald DJ, Pastan I

Cancer Res 1991 Jun 51:2808-12

BROWSE : Cancer Res • Volume 51 • Issue 11

VIEW : **MEDLINE**, full MEDLINE, related records

Abstract

TGF-alpha-PE40 is a chimeric protein composed of transforming growth factor alpha (TGF-alpha) linked to a modified Pseudomonas toxin from which the cell recognition domain has been deleted (PE40). TGF-alpha-PE40 has been shown to have cytotoxic effects on human cancer cell lines that express the epidermal growth factor (EGF) receptor on their surface, and when given i.p., it prolongs the survival of nude mice bearing i.p. tumors. Because several normal tissues, including liver, express EGF receptors on their surfaces, it has not been clear that this agent can be used systemically to treat EGF receptor-bearing tumors. In this study, we have delivered TGF-alpha-PE40 for 7 days by continuous infusion through a miniosmotic pump placed in the peritoneal cavity of nude immunodeficient mice. Two different human cancer cell lines that express EGF receptors on their surface were implanted s.c. One was A431, an epidermoid carcinoma; the other was DU-145, a prostate carcinoma. By using this mode of continuous i.p. delivery, we were able to achieve a constant serum level of TGF-alpha-PE40 that was nontoxic to the mice and yet delayed the growth of both tumors implanted s.c. and caused partial regression of one. We conclude that it is possible to deliver TGF-alpha-PE40 systemically and achieve a therapeutic serum level in mice without major toxicity. Although side effects may be expected, this study establishes that there is a therapeutic window for this agent in the therapy of cancers with high numbers of EGF receptors.

MeSH

Animal; Carcinoma, Squamous Cell; Drug Screening Assays, Antitumor; Drug Stability; Exotoxins; Female; Infusion Pumps; Male; Mice; Mice, Nude; Prostatic Neoplasms; Recombinant Fusion Proteins; Transforming Growth Factor alpha; Tumor Cells, Cultured

Author Address

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Kreitman

Medline

Purification and characterization of IL6-PE4E, a recombinant fusion of interleukin 6 with Pseudomonas exotoxin.

Kreitman RJ, Pastan I

Bioconjug Chem 4:581-5

BROWSE : Bioconjug Chem • Volume 4 • Issue 6

VIEW : MEDLINE, full MEDLINE, related records

Abstract

We have developed a procedure to purify the recombinant fusion toxin IL6-PE4E from Escherichia coli which results in a high yield of fully active monomeric protein of high purity and very low endotoxin content. The chimeric toxin is composed of human interleukin 6 (IL6) fused to a derivative of Pseudomonas exotoxin (PE) containing mutations in the binding domain which prevent binding to the PE receptor. In a typical preparation, 20 g of E. coli cells expressing the plasmid encoding IL6-PE4E were treated with lysozyme and washed repeatedly with detergent (Triton X-100), to obtain 500 mg of inclusion bodies. The recombinant protein was denatured and reduced in guanidine hydrochloride solution containing dithioerythritol and refolded in a redox buffer containing oxidized glutathione and L-arginine. After purification of the dialyzed protein by anion-exchange, polymyxin B, and sizing chromatography, we obtained 100 mg (20% of recombinant protein) of purified monomer with 0.6-2.5 endotoxin units/mg of protein. Amino terminal sequencing confirmed the first 20 amino acids. IL6-PE4E purified in this manner was fully cytotoxic toward human multiple myeloma, hepatoma, epidermoid carcinoma, and prostate carcinoma cell lines. After intravenous injection into mice, we found the dose-limiting toxicity to be to the liver, by measurement of serum transaminases and histologic evaluation of the liver. The LD50 was 450 micrograms/kg. We conclude that IL6-PE4E can be purified efficiently for preclinical testing.

MeSH

Animal; Cells, Cultured; Comparative Study; Dose-Response Relationship, Drug; Escherichia coli; Exotoxins; Female; Human; Interleukin-6; Lethal Dose 50; Mice; Mice, Nude; Mutation; Recombinant Fusion Proteins

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Puri

Medline

A chimeric protein comprised of IL-4 and Pseudomonas exotoxin is cytotoxic for activated human lymphocytes.

Puri RK, Mehrotra PT, Leland P, Kreitman RJ, Siegel JP, Pastan I

J Immunol 1994 Apr 152:3693-700

BROWSE : J Immunol • Volume 152 • Issue 7

VIEW : MEDLINE, full MEDLINE, related records

Abstract

IL4-Pseudomonas exotoxin (IL4-PE4E) is a chimeric molecule in which human IL-4 is genetically fused to the mutated binding domain of Pseudomonas exotoxin. This molecule binds specifically to human IL-4 receptor-bearing cells. IL4-PE4E was extremely cytotoxic to highly purified anti-CD3-activated CD8+ T lymphocytes. The cytotoxic activity of this molecule was dependent on the activation state of CD8+ T cells: 3- and 4-day activated T cells were very susceptible to the cytotoxic activity of IL4-PE4E compared with 0- to 2-day activated cells. PHA-activated lymphocytes and PBL activated in mixed lymphocyte reaction were also highly sensitive to IL4-PE4E. CD16+ and/or CD56+ highly purified NK cells or highly purified, IL-2-activated NK cells were also very sensitive to the cytotoxic effect of IL4-PE4E. IL-2-activated LAK cells had little susceptibility after 1 day but were very sensitive to IL4-PE4E after 3 days. The cytotoxic effects of IL4-PE4E were mediated through a ligand receptor interaction because excess rIL-4 abrogated these effects as did a neutralizing Ab to human IL-4. A chimeric mutant protein that can bind to IL-4 receptors but lacks the ability to inhibit protein synthesis was not cytotoxic to activated lymphocytes. The IL4-PE4E-mediated cytotoxicity of activated T cells correlated with the level of expression of IL-4 receptors on these cells. CD8+ T cells activated for 3 days expressed the highest density of IL-4 receptors compared with 1- or 2-day activated cells. Among two chimeric toxins tested only IL4-PE4E was cytotoxic to 2-day anti-CD3-activated CD8+ T lymphocytes, whereas IL6-PE4E was not active at all. These studies suggest that human IL4 toxin could be a potent agent for the elimination of activated lymphocytes in allograft rejection, some autoimmune diseases, or treatment of lymphomas and leukemias.

MeSH

Antigens, CD8; Chimeric Proteins; Comparative Study; Exotoxins; Interleukin-4; Isoantigens; Killer Cells, Lymphokine-Activated; Killer Cells, Natural; Lymphocyte Subsets; Lymphocyte Transformation; Phytohemagglutinins; Receptors, Interleukin-4; Receptors, Mitogen; Structure-Activity Relationship

Author Address

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Siegall

Medline

In vivo activities of acidic fibroblast growth factor-Pseudomonas exotoxin fusion proteins.

Siegall CB, Gawlak SL, Chace DF, Merwin JR, Pastan I

Bioconjug Chem 5:77-83

BROWSE : Bioconjug Chem • Volume 5 • Issue 1

VIEW : MEDLINE, full MEDLINE, related records

Abstract

Fibroblast growth factor receptors are highly expressed in a variety of cancer cells and activated vasculature. Using chimeric toxins targeted to cell-surface a FGF receptors, we have demonstrated specific cytotoxic activity to these cell types. These molecules, aFGF-PE40 and aFGF-PE40 KDEL, are fusion proteins containing acidic FGF and either a 40- or a 66-kDa binding defective form of Pseudomonas exotoxin, respectively. Both aFGF-toxin fusion proteins were able to inhibit protein synthesis in vitro in a variety of carcinoma cell lines. The half-life of aFGF-PE40 in serum was found to be 41 min when coadministered with heparin. Administration of aFGF-PE40 or aFGF-PE4E KDEL with heparin inhibits the growth of established KB and preestablished A431 epidermoid carcinoma xenografts in athymic mice. The antitumor activities of the two aFGF-toxin fusion proteins were equivalent against the KB tumor xenografts. While we were able to slow the growth of the KB tumor xenografts, we were unable to cause tumor regressions. Histochemical analysis of treated versus untreated tumor tissue revealed a difference in tumor size but not of vascularity. We conclude that aFGF-PE40 and aFGF-PE4E KDEL have in vivo antitumor activity that targets the tumor cell mass rather than vascular structures in mice xenografted with human epidermoid carcinoma.

MeSH

Animal; Antineoplastic Agents; Comparative Study; Drug Carriers; Exotoxins; Fibroblast Growth Factor, Acidic; Histocytochemistry; Human; Mice; Mice, Inbred BALB C; Mice, Nude; Neoplasm Proteins; Neoplasm Transplantation; Neoplasms, Experimental; Receptors, Fibroblast Growth Factor; Recombinant Fusion Proteins; Transplantation, Heterologous

Author Address

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Mansfield

Medline

Characterization of RFB4-Pseudomonas exotoxin A immunotoxins targeted to CD22 on B-cell malignancies.

Mansfield E, Pastan I, FitzGerald DJ

Bioconjug Chem 7:557-63

BROWSE : Bioconjug Chem • Volume 7 • Issue 5

VIEW : **MEDLINE**, full MEDLINE, related records

Abstract

To develop an immunotoxin for the treatment of B-cell malignancies, we constructed several candidate conjugates with RFB4, a B-cell specific anti-CD22 IgG1, and truncated forms of Pseudomonas exotoxin (PE). The four versions of PE included PE35 and PE35KDEL, which were linked to RFB4 via a disulfide bond, and PE38 and PE38KDEL, which were linked via a thioether bond. The PE35 truncated forms, which are fully active in ADP ribosylation and lack receptor binding sequences, do not require intracellular proteolytic cleavage in order to be active. PE35KDEL has the consensus endoplasmic reticulum retention signal, KDEL, replacing the wild type PE C-terminal sequence, REDLK. The PE38 forms retain all of domain II and therefore require cleavage to be active within cells. Cytotoxicity experiments on CD22-positive cell lines revealed that the PE35 conjugates were more active than the PE38 versions and the presence of the KDEL sequence generally enhanced toxicity by 5-10-fold compared to that of REDLK. The RFB4-PE35KDEL immunotoxin was most active in cytotoxicity assays against Burkitt's lymphoma cell lines such as Daudi and CA46 (IC50 = 0.2 ng/mL) and displayed little cytotoxicity toward human vascular endothelial cells (IC50 > 20 micrograms/mL). Results of experiments conducted in nude mice showed that both RFB4-PE35KDEL and RFB4-PE35 could inhibit the development of subcutaneous CA46 tumors.

MeSH

Adenosine Diphosphate Ribose; Animal; Antigens, CD; Antigens, Differentiation, B-Lymphocyte; Bacterial Toxins; Cell Survival; Endothelium, Vascular; Exotoxins; Human; Immunotoxins; Lymphoma, B-Cell; Mice; NAD+ ADP-Ribosyltransferase; Pseudomonas aeruginosa; Tumor Cells, Cultured

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Order Document

Kuan

Medline

Immunotoxins containing Pseudomonas exotoxin that target LeY damage human endothelial cells in an antibody-specific mode: relevance to vascular leak syndrome.

Kuan CT, Pai LH, Pastan I

Clin Cancer Res 1995 Dec 1:1589-94

BROWSE : Clin Cancer Res • Volume 1 • Issue 12

VIEW : MEDLINE, full MEDLINE, related records

Abstract

Vascular leak syndrome (VLS) was originally found to be a major dose-limiting toxicity in humans with cancer treated with several immunotoxins (ITs) containing ricin A chain or blocked ricin. Recently, VLS has also been observed in patients treated with an IT containing the murine monoclonal antibody (MAb) B3 coupled to LysPE38, a recombinant truncated form of Pseudomonas exotoxin (PE) A. Antibody B3 (IgG1k) recognizes LewisY and related carbohydrate epitopes present on many human solid tumors, and B3-LysPE38 showed excellent antitumor activity in nude mice bearing tumors that express the B3 antigen. In the clinical trial, the development of VLS has prevented the administration of the amount of IT necessary to achieve blood levels required for good therapeutic responses. We have now investigated the effects of several PE-based ITs on different human endothelial cell lines to elucidate the mechanism of VLS induced by ITs containing PE. To assess the cytotoxic effect of IT on endothelial cells, various ITs were incubated with cells for 2 or 20 h, and the incorporation of [3H]leucine into protein was measured. The endothelial cells studied were human umbilical vein endothelial cells, human lung-derived microvascular endothelial cells (HUVECs), human adult dermal microvascular endothelial cells, human pulmonary artery endothelial cells, and human aortic endothelial cells. We found that both B3-LysPE38 (LMB-1), a chemical conjugate of MAb B3 with PE38, as well as B3(Fv)-PE38 (LMB-7), a recombinant single chain immunotoxin, inhibited protein synthesis, with 50% inhibitory concentrations between 600 and 1000 ng/ml for 20-h incubation in HUVECs, human lung-derived microvascular endothelial cells, and human adult dermal microvascular endothelial cells but not on human pulmonary artery endothelial cells. The cytotoxic effect was specific since PE38 itself or PE coupled to several other antibodies did not inhibit protein synthesis in these cells even at 10,000 ng/ml. Further evidence that the cytotoxicity of B3-containing ITs is due to specific B3 binding to endothelial cells comes from the fact that the cytotoxicity can be blocked by excess free MAb B3. HUVECs undergo overt morphological changes after treatment with B3-LysPE38 or B3(Fv)PE38. Gaps between the cells are formed after a 20-h exposure but not after 2 h. These studies suggest that VLS in patients is due to capillary damage caused by prolonged exposure to high concentrations of LMB-1.

MeSH

Antibodies, Monoclonal; Antibody Specificity; Capillary Leak Syndrome; Cells, Cultured; Endothelium, Vascular; Exotoxins; Human; Immunotoxins; Lewis Blood-Group System; Proteins

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Order Document

Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 974–978, February 1996
Immunology

Improved antitumor activity of a recombinant anti-Lewis^y immunotoxin not requiring proteolytic activation

(cancer therapy/*Pseudomonas* exotoxin/monoclonal antibody B1 Fv fragment/disulfide-stabilized Fv fragment/protein engineering)

CHIEN-TSUN KUAN AND IRA PASTAN*

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Contributed by Ira Pastan, October 16, 1995

ABSTRACT **B1(dsFv)-PE33 is a recombinant immunotoxin composed of a mutant form of *Pseudomonas* exotoxin (PE) that does not need proteolytic activation and a disulfide-stabilized Fv fragment of the anti-Lewis^y monoclonal antibody B1, which recognizes a carbohydrate epitope on human carcinoma cells. In this molecule, amino acids 1–279 of PE are deleted and domain Ib (amino acids 365–394) is replaced by the heavy chain variable region (V_H) domain of monoclonal antibody B1. The light chain (V_L) domain is connected to the V_H domain by a disulfide bond. This recombinant toxin, termed B1(dsFv)-PE33, does not require proteolytic activation and it is smaller than other immunotoxins directed at Lewis^y, all of which require proteolytic activation. Furthermore, it is more cytotoxic to antigen-positive cell lines. B1(dsFv)-PE38 has the highest antitumor activity of anti-Lewis^y immunotoxins previously constructed. B1(dsFv)-PE33 caused complete regression of tumors when given at 12 μg/kg (200 pmol/kg) every other day for three doses, whereas B1(dsFv)-PE38 did not cause regressions at 13 μg/kg (200 pmol/kg). By bypassing the need for proteolytic activation and decreasing molecular size we have enlarged the therapeutic window for the treatment of human cancers growing in mice, so that complete remissions are observed at 2.5% of the LD_{50}.**

Recombinant Fv-immunotoxins are chimeric proteins in which a truncated toxin is fused to an Fv fragment of an antibody. The Fv region targets antigens on tumor cells and the toxin moiety kills the cell. Fv-immunotoxins have very good cytotoxic activity on human tumor cell lines and can cause complete regression of established human tumor xenografts in mice (1–3). Several Fv-immunotoxins are currently being evaluated in clinical or preclinical trials (4). Originally, the Fv fragments of the recombinant toxins were designed in a single-chain form (scFv-immunotoxins), in which the heavy and light chain variable region (V_H and V_L) domains are connected by a flexible peptide linker (5, 6). Subsequently a method was developed to stabilize the Fv fragments by an interchain disulfide bond that connects structurally conserved framework regions of the V_H and V_L domains (refs. 7–9 and reviewed by Reiter and Pastan in ref. 10). Such disulfide-stabilized dsFv-immunotoxins are much more stable than scFv-immunotoxins, and some have improved antigen-binding affinities and improved antitumor activities (11). A major advantage of using Fv fragments, which are the smallest functional modules of antibodies, in recombinant immunotoxins is that these molecules are significantly smaller than chemical conjugates made with whole antibodies. This allows them to effectively penetrate into solid tumors (12, 13).

Pseudomonas exotoxin (PE)-based recombinant immunotoxins require proteolytic activation. Domain II of the toxin is cleaved between amino acids 279 and 280, a reaction that is catalyzed by the enzyme furin (14, 15). This step could be rate limiting, because the furin concentration in cells is low and some cancer cells may be furin deficient. Therefore, we have constructed a recombinant immunotoxin that does not need proteolytic activation. Furin cleavage generates a 37-kDa carboxyl-terminal fragment of PE, amino acids 280–613 (PE37), that contains the translocating and ADP-ribosylation activity of PE (16, 17). If a functional Fv fragment could be inserted into PE37 without destroying its ADP-ribosylation activity or translocating ability and the Fv still retained its binding affinity, then the recombinant molecule should be more active than a toxin which needs to be proteolytically processed.

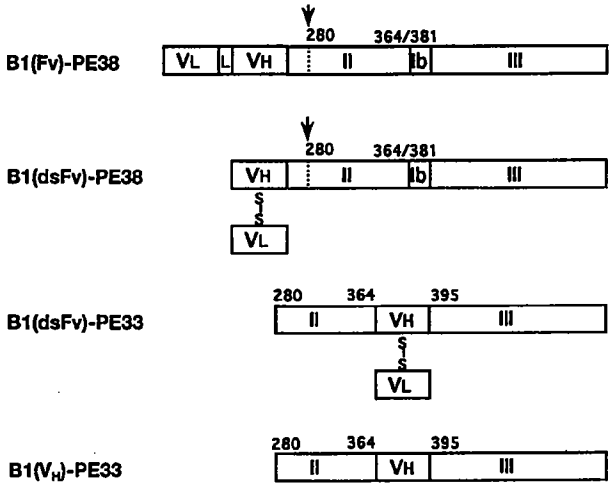

FIG. 1. Schematic of expressed proteins. Positions of amino acids that span PE sequences are numbered. The arrow marks the proteolytic site of PE for activation. S—S shows the disulfide bond linkage between the Fv fragments. L, peptide linker; II, PE domain II for translocation; Ib, PE domain Ib (function unknown); III, PE domain III for ADP-ribosylation of EF2.

Monoclonal antibody (mAb) B1 is a murine antibody directed against Lewis^y-related carbohydrate antigens, which are abundant on the surface of many carcinomas (18). mAb B1 has been used to make both single-chain and disulfide-stabilized Fv-immunotoxins (18–20). These agents are capable of causing complete regression of established xenografts in nude mice (20). To develop a recombinant immunotoxin that is small and stable and does not need proteolytic processing, we have replaced domain Ib (amino acids 365–394) of PE37 with the

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Abbreviations: mAb, monoclonal antibody; V_H and V_L, variable heavy and light chain, respectively; scFv and dsFv, single-chain and disulfide-stabilized Fv, respectively; PE, *Pseudomonas exotoxin*; Mes, 4-morpholineethanesulfonic acid.

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V_H fragment of mAb B1 and linked the V_H domain to the V_L domain with a disulfide bond (Fig. 1). We find that the resulting molecule, B1(dsFv)-PE33, is more active than any previous mAb B1-containing immunotoxin.

MATERIALS AND METHODS

Construction of Plasmids for Expression of B1(dsFv)-PE33. "Sticky feet"-directed mutagenesis (21) using uracil-containing pDF1 as a template was used to construct the expression plasmid encoding for B1(V_H)R44C-PE33, the component of the intramolecularly inserted dsFv-immunotoxin. The construction of plasmids pDF1, which encodes PE37, which starts at methionine followed by PE amino acids 281–613 (a truncated form of PE that does not require proteolytic activation), and pB1V_HR44C-PE38, which encodes the single-domain B1(V_H)R44C-PE38 immunotoxin, has been described (16, 20). The B1(V_H)R44C DNA was PCR amplified by using the plasmid pB1V_HR44C-PE38 as a template and oligo primers CT119 and 5′-phosphorylated CT120. The forward PCR primer CT119, 5′-GGCAACGACGAGGCCGGCGCGGCCAACGGCGGTGGCGGATCCGAGGTGCAGCTGGTGGAATCTGGA-3′, had sequences that are identical to the DNA encoding PE residues 356–364 and a peptide linker GGGGS inserted at the 5′ end of V_H, and a *Bam*HI site was created (underlined). The reverse PCR oligonucleotide primer CT120, 5′-GTCGCCGAGGAACTCCGCGCCAGTGGGCTCGGGACCTCCGGAAGCTTTTGC-3′, had sequences that are complementary to the DNA encoding PE residues 395–403 and an Fv-toxin junction (connector) inserted at the 3′ end of V_H, and a *Hin*dIII site was created (underlined). The PCR product was purified and annealed with a uracil-containing single-stranded DNA prepared by the rescue of pDF1 phagemid with an M13K07 helper phage (Bio-Rad). The DNA was extended and ligated according to the Muta-Gene mutagenesis kit (Bio-Rad). Because the annealing efficiency of the PCR fragment to the single-stranded template and the mutagenesis efficiency were low (≈10%), the DNA pool of the mutagenesis reaction was digested with a restriction endonuclease which cuts a unique site in the domain Ib region but not in B1(V_H). This extra digestion step improved the mutagenesis efficiency to more than 50%. Correct clones were identified by DNA restriction analysis and verified by DNA sequencing. The resulting immunotoxin clone was named pB1(V_H)R44C-PE33 or pCTK104. It encodes a single-domain B1(V_H)-immunotoxin in which the V_H domain replaces the domain Ib region (amino acids 365–394) of PE37. The plasmid pB1V_LA105CSTOP encodes B1(V_L)A105C, which is a component of dsFv-immunotoxin as described previously (20).

Production of Recombinant Immunotoxin. The components of the disulfide-stabilized immunotoxins B1(V_H)R44C-PE38, B1(V_H)R44C-PE33, and B1(V_L)A105C or the single-chain immunotoxin B1(Fv)-PE38 were produced in separate *Escherichia coli* BL21(λDE3) (22) cultures harboring the corresponding expression plasmid. All recombinant proteins accumulated in inclusion bodies. Disulfide-stabilized immunotoxins were obtained by mixing equimolar amounts of solubilized and reduced inclusion bodies essentially as described (23), except that the final oxidation step was omitted and refolding was carried out at pH 9.5. Properly folded disulfide-stabilized and single-chain immunotoxins were purified by sequential ion-exchange (Q-Sepharose and Mono Q) followed by size-exclusion chromatography on a TSK G3000SW (TosoHaas) column as described (7).

Analysis of Immunotoxins. The cytotoxic activity of immunotoxins was determined by inhibition of protein synthesis as described (24). For competition assays designed to prove the specificity of the recombinant immunotoxins, we changed the medium and added 50 μg of antibody per well 30 min prior to the addition of the immunotoxin. Thermal stability of the immunotoxins was determined by incubating them at 100 μg/ml in phosphate-buffered saline (PBS; 6.7 mM sodium phosphate, pH 7.4/150 mM NaCl) at 37°C for 8 h, followed by analytical chromatography on a TSK G3000SW (TosoHaas) column to separate the monomers from larger aggregates (8). Relative binding affinities of the immunotoxins were determined by adding ^{125}I-labeled B1-IgG to 10^5 A431 cells as a tracer with various concentrations of the competitor. The binding assays were performed at 4°C for 2 h in RPMI medium 1640 containing 1% bovine serum albumin and 50 mM Mes (Sigma) as described (25).

Toxicity and Antitumor Activity in Nude Mice. The single-dose mouse LD_{50} was determined by using female BALB/c mice (6–8 weeks old, ≈20 g), which were given a single i.v. injection of different doses of B1(dsFv)PE38 or B1(dsFv)PE33 diluted in 200 μl of PBS containing 0.2% human serum albumin (PBS-HSA). Mice were followed for 2 weeks after injection. Athymic (*nu/nu*) mice, females 6–8 weeks old, ≈20 g, were injected s.c. on day 0 with 3×10^6 A431 cells suspended in RPMI medium without fetal bovine serum. By day 5, tumors were about 50–70 mm^3 in size. Mice were treated on days 5, 7, and 9 by i.v. injections of different doses of immunotoxins diluted in PBS-HSA. Tumors were measured with a caliper and the tumor volumes were calculated by using the formula volume = length × width^2 × 0.4.

RESULTS

Plasmid Constructions and Production of B1(dsFv)-PE33. Our goal was to construct an active recombinant immunotoxin that was smaller than the current generation of recombinant immunotoxins and that did not need intracellular proteolytic cleavage for activation. To do this we inserted the B1 dsFv fragment between domains II and III by replacing domain Ib of PE37, a truncated form of PE that contains only the portion of the toxin that undergoes translocation to the cytosol. As shown in Fig. 1, the V_H domain replaces amino acids 365–394 of PE37 and the V_L domain is connected to the V_H domain by a disulfide bond engineered into the framework region, with cysteines introduced at position 44 of V_H and position 105 of V_L (7). The resulting recombinant immunotoxin, termed B1(dsFv)-PE33, is 5 kDa smaller than B1(dsFv)-PE38 or B1(Fv)-PE38 (Fig. 1). In the toxin portion, Cys-287 was changed to a Ser to reduce the chance of incorrect disulfide bond formation (26). B1(V_H)R44C is inserted after amino acid 364 of PE and the insert is preceded by a small flexible peptide linker, GGGGS. Following the V_H domain is another peptide, KASGGPE, C3 connector (27), that connects the carboxyl terminus of V_H to amino acid 395 of PE.

The "sticky feet"-directed mutagenesis protocol used for the construction of B1(V_H)R44C-PE33 is described in *Materials and Methods*. Immunotoxins were expressed in *E. coli* BL21(λDE3); cultures for expressing the components of the dsFv-immunotoxin were prepared separately. The immunotoxins were purified by refolding of inclusion bodies in a redox-shuffling buffer and sequential ion-exchange and gel-filtration chromatography as described in *Materials and Methods*. The proteins obtained were more than 95% homogeneous and had the expected molecular mass of 59 kDa on SDS/PAGE as shown in lane 2 of Fig. 2. In the presence of the reducing agent 2-mercaptoethanol, the dsFv-immunotoxin, B1(dsFv)-PE33, dissociated into its two components (lane 4), B1(V_L) and B1(V_H)-PE33. The apparent molecular masses of these components are 13 kDa and 46 kDa, respectively. We also produced the single-domain B1(V_H)-PE33 immunotoxin as shown in Fig. 2. The yield of either B1(dsFv)-PE33 or B1(V_H)-PE33 was 8–10% of the total protein present in inclusion bodies.

Improved Cytotoxic Activity of B1(dsFv)-PE33 Toward B1-Antigen-Expressing Cell Lines. The cytotoxicity of B1(dsFv)-

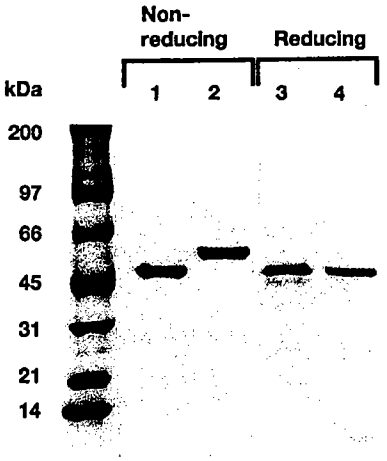

FIG. 2. Purity of B1(dsFv)-PE33 and B1(V_H)-PE33: SDS/4–20% PAGE. Lanes: 1, B1(V_H)-PE33, nonreduced; 2, B1(dsFv)-PE33, nonreduced; 3, B1(V_H)-PE33, reduced; and 4, B1(dsFv)-PE33, reduced. The left lane contains mass markers.

PE33 was determined by measuring the decrease in incorporation of [^{3}H]leucine by various human cancer cell lines after treatment with immunotoxin (24). B1(dsFv)-PE38 and B1(V_H)-PE33 (no light chain) were included for comparison. Fig. 3*A* and Table 1 show that all three proteins are cytotoxic to cells expressing B1 antigen (A431, MCF7, CRL1739, and LNCaP) but not to cells that do not bind mAb B1 (L929 and HUT102). In this assay, B1(dsFv)-PE33 had an IC_{50} of 0.25 ng/ml on A431 cells and 0.35 ng/ml on MCF7 cells. We found

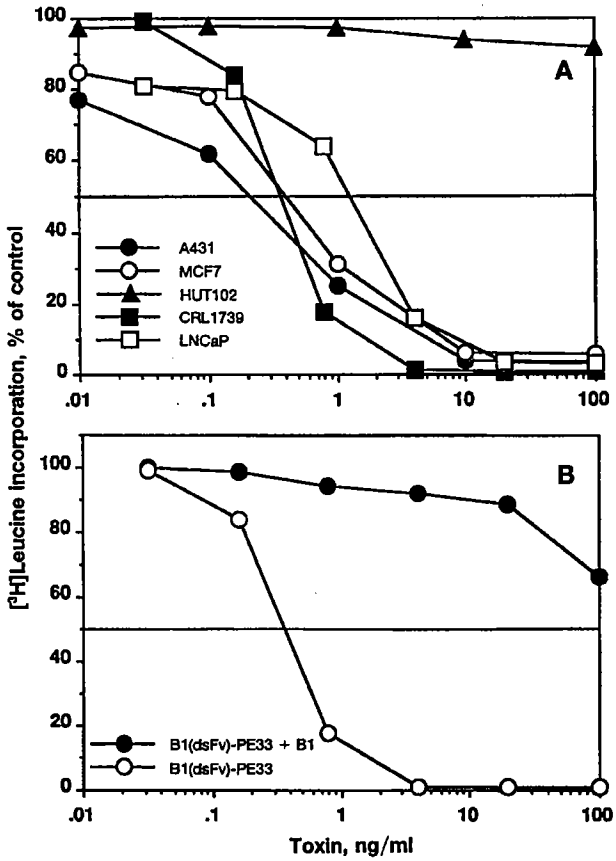

FIG. 3. (*A*) Toxicity of B1(dsFv)-PE33 for various cell lines. (*B*) mAb B1 inhibition of the cytotoxicity of B1(dsFv)-PE33 for A431 cells.

Table 1. Cytotoxicity of B1 immunotoxins toward various cell lines

Cell line*	Cancer type	Antigen expression†	IC_{50},‡ ng/ml B1(dsFv)-PE38	B1(dsFv)-PE33	B1(V_H)-PE33
A431	Epidermoid	+++	0.5	0.25	2.0
MCF7	Breast carcinoma	+++	0.9	0.35	4.0
CRL1739	Gastric	+++	0.4	0.31	ND
LNCaP	Prostate	+	7.0	1.3	ND
HUT102	T-cell leukemia	–	>1000	>1000	>1000
L929	Mouse fibroblast	–	>1000	>1000	>1000

ND, not determined.
*All the cell lines except L929 are of human origin.
†The level of antigen expression is marked +++, +, and – for strong, medium, and no detectable expression in immunofluorescence, respectively.
‡Cytotoxicity data are given as IC_{50} value, the concentration of immunotoxin that causes a 50% inhibition of protein synthesis after a 20-h incubation with immunotoxin.

that B1(dsFv)-PE33 was more active on all antigen-positive cell lines compared with B1(dsFv)-PE38, which requires proteolytic processing. To analyze whether the cytotoxicity of B1(dsFv)-PE33 was specific, competition experiments were carried out with an excess of mAb B1. Fig. 3*B* shows that the intoxication of A431 carcinoma cells by B1(dsFv)-PE33 is due to the specific binding to the B1 antigen, since its cytotoxicity was blocked by excess mAb B1. We also tested B1(V_H)-PE33, which is not associated with light chain, and found that it was only about 10-fold less cytotoxic (IC_{50} of 2 ng/ml on A431 cells) than B1(dsFv)-PE33 (Table 1), indicating the heavy chain has a major role in antigen binding. However, a related single-domain immunotoxin, B3(V_H)-PE38, which requires proteolytic processing for activation, is much less active, with an IC_{50} of 40 ng/ml on A431 cells (28).

Antigen Binding of B1(dsFv)-PE33. To determine whether the improved cytotoxicity of B1(dsFv)-PE33 is due to improved binding, we analyzed its binding affinity to antigen-positive cells (A431 cells) by competition assays, in which increasing concentrations of each immunotoxin competed for the binding of ^{125}I-labeled B1 IgG to A431 cells at 4°C. The results shown in Fig. 4 indicate that B1 IgG, B1(dsFv)-PE38, B1(dsFv)-PE33, and B1(V_H)-PE33 competed for the binding of ^{125}I-labeled B1 IgG to A431 cells by 50% at 40 nM, 2 μM, 3.5 μM, and 25 μM, respectively. Thus, the binding affinity of

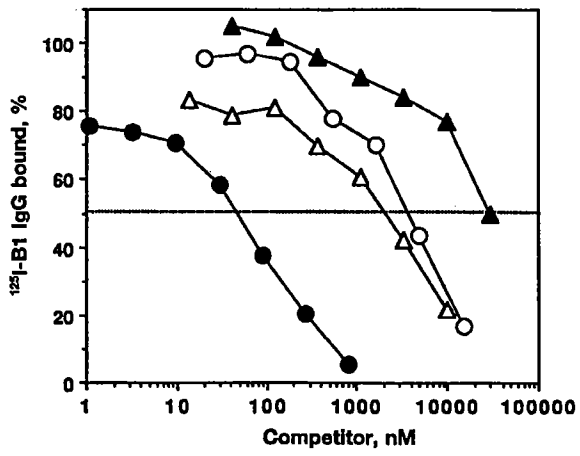

FIG. 4. Binding of B1(dsFv)-PE33, B1(dsFv)-PE38, and B1(V_H)-PE33 to A431 cells: Competitive binding analysis of the ability of purified B1(dsFv)-PE33, B1(dsFv)-PE38, and B1(V_H)-PE33 to inhibit the binding of ^{125}I-labeled B1 IgG to A431 cells overexpressing B1 antigen. ●, B1 IgG; ○, B1(dsFv)-PE33; △, B1(dsFv)-PE38; and ▲, B1(V_H)-PE33.

Table 2. Thermal stability of immunotoxins

Immunotoxin	Amount, % of control	
	Monomer	Aggregates
B1(dsFv)-PE38	100	<0.1
B1(Fv)-PE38	≈40	≈60
B1(dsFv)-PE33	100	<0.1

The thermal stability of immunotoxins to heat treatment was determined by incubation at 0.1 mg/ml in PBS at 37°C for 8 h, followed by analytical chromatography on a TSK G3000SW (TosoHaas) column, to quantitate the amount of monomers and aggregates compared to the untreated immunotoxins.

B1(dsFv)-PE33 is slightly reduced compared with B1(dsFv)-PE38. Therefore, the improved cytotoxicity cannot be due to improved binding, suggesting that elimination of the requirement for proteolytic activation is probably responsible for the improved cytotoxicity. The single-domain immunotoxin B1(V_H)-PE33 exhibited a 10-fold lower binding affinity relative to the dsFv-immunotoxins, which is consistent with its diminished cytotoxicity (Table 1).

Stability of Immunotoxin. The stability of immunotoxins at 37°C is an important factor in their usefulness as therapeutic agents. The stability of an immunotoxin is governed by its tendency to aggregate at 37°C. The thermal stability of B1(dsFv)-PE33, B1(dsFv)-PE38, and B1(Fv)-PE38 was determined by measuring the amount of aggregation and inactivation at 37°C as described in *Materials and Methods.* We found that both B1(dsFv)-PE33 and B1(dsFv)-PE38 were monomers before incubation in PBS at 37°C and remained monomeric for 8 h (Table 2). In contrast, the single-chain immunotoxin B1(Fv)-PE38 formed >60% aggregates after an 8-h incubation at 37°C (Table 2; also see ref. 20). Following the 8-h incubation at 37°C, B1(dsFv)-PE33 and B1(dsFv)-PE38 retained almost all their initial cytotoxic activity, while B1(Fv)-PE38 lost 75% of its initial cytotoxic activity (20). Thus, both B1(dsFv)-PE38 and B1(dsFv)-PE33 are extremely stable at 37°C, presumably because they do not tend to aggregate as do the scFv-immunotoxins.

Toxicity of Immunotoxins in Mice. The toxicity of single doses of the immunotoxins B1(dsFv)-PE33 and B1(dsFv)-PE38 was measured by i.v. injections of different amounts of immunotoxin into BALB/c mice. The mice were observed for 14 days after injection. The LD_{50} of both immunotoxins was found to be 0.5 mg/kg, similar to the LD_{50} determined for the B1(dsFv)-PE38 as well as other anti-Lewis^y Fv-immunotoxins (23). The results show that even though the immunotoxin is more active on target cells because it does not require proteolytic activation, it is not more toxic to mice. This toxicity in mice is presumed to be due to nonspecific uptake of the toxin moiety by the liver (29).

Improved Antitumor Activity of B1(dsFv)-PE33. To determine whether the improved cytotoxicity *in vitro* is accompanied by an increase in antitumor activity, B1(dsFv)-PE33 and B1(dsFv)-PE38 were compared by assessing their ability to cause regression of established human carcinoma xenografts in nude mice. Nude mice were injected with 3 × 10^6 A431 cells s.c. on day 0. Beginning 5 days later, when tumors averaged

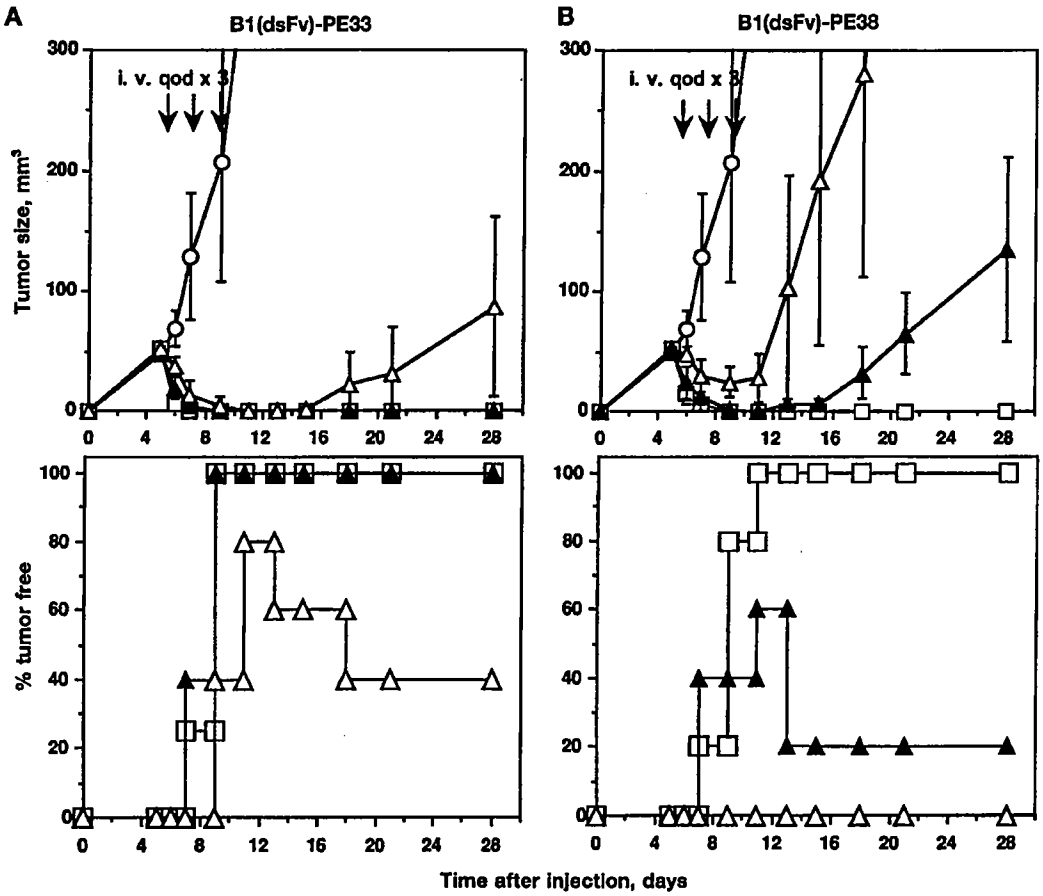

FIG. 5. Antitumor effect and durability of complete remissions due to B1(dsFv)-PE33 and B1(dsFv)-PE38 in a nude mouse model. Groups of five mice were injected s.c. with 3 × 10^6 A431 cells on day 0 and were treated by i.v. injections of B1(dsFv)-PE33 (*Left*) or B1(dsFv)-PE38 (*Right*) on days 5, 7, and 9 (indicated by arrows), when the tumors were established. Control mice were treated with PBS-HSA. Error bars represent the standard error of the data. ○, Control; □, 400 pmol/kg; ▲, 200 pmol/kg; and △, 100 pmol/kg.

50–70 mm^3 in volume, the mice were treated with i.v. injections on days 5, 7, and 9 of various doses of immunotoxin. Control mice were treated with PBS-HSA only. As shown in Fig. 5, both immunotoxins demonstrated significant dose-dependent antitumor activity. B1(dsFv)-PE38 caused only partial regression of A431 tumors at the 6.5 μg/kg (100 pmol/kg) dose level, whereas B1(dsFv)-PE33 at the same dose caused complete disappearance of the tumors (Fig. 5). Furthermore, the tumors treated with B1(dsFv)-PE38 at 200 pmol/kg (13 μg/kg) regressed completely after the third injection but regrew within a few days, whereas B1(dsFv)-PE33 at 200 pmol/kg (12 μg/kg) caused complete regressions that lasted over 1 month in 5 of 5 animals. These results indicate that B1(dsFv)-PE33 has significantly better antitumor activity than B1(dsFv)-PE38. Thus, the improved cytotoxicity *in vitro* results in improved antitumor activity in animals.

DISCUSSION

We have developed a recombinant immunotoxin composed of a dsFv fragment of mAb B1 and a truncated form of PE that is smaller than other recombinant PE-derived immunotoxins and does not require intracellular proteolytic activation. Another advantage is that it is more active *in vitro* and a more potent antitumor agent than immunotoxins made with the same antibody that require proteolytic processing (20).

Location for B1(dsFv) Insertion in PE33. The B1 dsFv fragment was inserted between the translocation domain and ADP-ribosylation domain of PE, replacing domain Ib. The rationale for this design is that domain Ib (amino acids 364–395) is not essential for the cytotoxic activity (30), and it can be completely deleted from immunotoxins without loss of activity. In fact, it is also possible to delete a portion of domain II (amino acids 343–364) without loss of activity. In addition, analysis of the proposed structure of B1(dsFv)-PE33 by using computer graphics (N. Kurochkina, C.-T.K. and I.P., unpublished results) indicates that domain Ib should be a good location for insertion of the dsFv fragment, because the complementarity-determining regions of the Fv should be "free" to interact with antigen. The results in Fig. 4 show that the presence of B1(dsFv) in this region only minimally affected antigen binding. In another study, we have inserted a dsFv fragment of mAb e23, which binds to the erbB2 antigen, near the carboxyl terminus of PE35 at amino acid 607; this location was found to significantly decrease antigen binding of e23(dsFv) to its antigen (31). It is necessary to investigate whether the domain Ib location is useful for the insertion of other dsFvs.

Improved Antitumor Activity of B1(dsFv)-PE33. To compare the antitumor activity of B1(dsFv)-PE33 with B1(dsFv)-PE38, we used the A431 human epidermoid carcinoma model to evaluate the ability of both immunotoxins to cause complete regression of tumors. B1(dsFv)-PE38 is very potent in antitumor activity (20). We found that when the nude mice were treated with three doses of 200 pmol/kg, given every other day, B1(dsFv)-PE38 caused significant tumor regressions but did not produce cures. In contrast, B1(dsFv)-PE33 caused complete remissions and cures in all animals at the same dose of 200 pmol/kg (Fig. 5). Thus, B1(dsFv)-PE33 has better antitumor activity than B1(dsFv)-PE38. Since both B1(dsFv)-PE33 and B1(dsFv)-PE38 have the same toxicity in mice, the PE33 immunotoxin has a larger therapeutic window. The effective dose causing complete remissions in nude mice is 2.5% of the LD_{50}. This makes B1(dsFv)-PE33 a good candidate for clinical development. The improved antitumor activity of B1(dsFv)-PE33 over B1(dsFv)-PE38 is a consequence of better cytotoxicity *in vitro* due to lack of requirement for proteolytic activation and possibly smaller size for better tumor penetration. Since the efficiency of proteolytic activation can vary in different types of cells, the type of recombinant immunotoxin described here may be more useful than the previous generation of molecules, which require proteolytic activation.

We thank I. Margulies and E. Lovelace for cell culture assistance, I. Benhar for his generous gifts of plasmids $pB1V_HR44C$-PE38 and $pB1V_LA105CSTOP$, and J. Evans and A. Jackson for editorial assistance. I.P. is the inventor on several patents related to this research which have been assigned to the National Institutes of Health.

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Choe

Medline

B3(Fab)-PE38M: a recombinant immunotoxin in which a mutant form of Pseudomonas exotoxin is fused to the Fab fragment of monoclonal antibody B3.

Choe M, Webber KO, Pastan I

Cancer Res 1994 Jul 54:3460-7

BROWSE : Cancer Res • Volume 54 • Issue 13

VIEW : **MEDLINE**, full MEDLINE, related records

Abstract

Recombinant immunotoxins were made by fusing the Fab domain of monoclonal antibody (MAb) B3 to PE38M, a truncated mutant form of Pseudomonas exotoxin (PE). The recombinant toxins were made in Escherichia coli by fusing genes encoding the antibody domains to a gene encoding the mutant form of PE. MAb B3 binds to a carbohydrate antigen found on many kinds of carcinomas. Immunotoxins in which MAb B3 has been chemically coupled to recombinant forms of PE have been shown to be very active cytotoxic agents. PE has also been targeted to tumor cells by replacing the cell-binding domain of PE (domain I) with a single-chain antibody to make a single-chain immunotoxin. In the current study, PE38M, a mutant form of PE, with a deletion of the cell-binding domain (amino acids 1-252) as well as mutations in domain III and some nonessential sequences in domain Ib (amino acids 365-380), was fused to the light chain of MAb B3. This protein was renatured in the presence of the Fd fragment of MAb B3 to produce a Fab-toxin fusion protein. Alternatively, the Fd fragment of MAb B3 was fused to PE38M and combined with the light chain. Both types of B3(Fab)-PE38M were just as active on target cells as previously described single-chain immunotoxins. Furthermore, the B3(Fab)-PE38M produced complete remissions of human tumor xenografts growing in nude mice. B3(Fab)-PE38M has two advantages over single-chain immunotoxins. One is that the yield of recombinant Fab-toxin is very high, with 10-22% of the starting protein recovered as cytotoxically active immunotoxin after chromatographic purification. The second is that the B3(Fab)-PE38M has a much longer survival in the circulation of mice with a t1/2 beta of approximately 5 h.

MeSH

Animal; Antibodies, Monoclonal; Base Sequence; Escherichia coli; Exotoxins; Half-Life; Immunoglobulins, Fab; Immunotoxins; Mice; Mice, Inbred BALB C; Molecular Sequence Data; Molecular Weight; Protein Structure, Secondary

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Pai

Medline

Treatment of advanced solid tumors with immunotoxin LMB-1: an antibody linked to Pseudomonas exotoxin.

Pai LH, Wittes R, Setser A, Willingham MC, Pastan I

Nat Med 1996 Mar 2:350-3

BROWSE : Nat Med • Volume 2 • Issue 3

VIEW : **MEDLINE**, full MEDLINE, related records

Abstract

Immunotoxin LMB-1 is composed of monoclonal antibody B3 chemically linked to PE38, a genetically engineered form of Pseudomonas exotoxin. B3 recognizes a carbohydrate antigen (Le (Y)) present on many human solid tumors. LMB-1 has excellent antitumor activity in nude mice bearing Le(Y)-positive tumors. We conducted a phase I study of 38 patients with solid tumors who failed conventional therapy and whose tumors expressed the Le(Y) antigen. Objective antitumor activity was observed in 5 patients, 18 had stable disease, 15 progressed. A complete remission was observed in a patient with metastatic breast cancer to supraclavicular nodes. A greater than 75% tumor reduction and resolution of all clinical symptoms lasting for more than six months was observed in a colon cancer patient with extensive retroperitoneal and cervical metastasis. Three patients (two colon, one breast cancer) had minor responses. The maximum tolerated dose of LMB-1 is 75 microgram/kg given intravenously three times every other day. The major toxicity is vascular leak syndrome manifested by hypoalbuminemia, fluid retention, hypotension and, in one case, pulmonary edema. Although immunotoxins have been evaluated in clinical studies for more than two decades, this is the first report of antitumor activity in epithelial tumors.

MeSH

Adult; Aged; Animal; Antibodies, Monoclonal; Drug Tolerance; Exotoxins; Female; Human; Immunotoxins; Lewis Blood-Group System; Male; Mice; Middle Age; Neoplasms; Tomography, X-Ray Computed; Vascular Diseases

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Order Document

Mickisch

Medline

Pseudomonas exotoxin conjugated to monoclonal antibody MRK16 specifically kills multidrug resistant cells in cultured renal carcinomas and in MDR-transgenic mice.

Mickisch GH, Pai LH, Siegsmund M, Campain J, Gottesman MM, Pastan I

J Urol 1993 Jan 149:174-8

BROWSE : J Urol • Volume 149 • Issue 1

VIEW : MEDLINE, full MEDLINE, related records

Abstract

Using renal carcinoma and prostate carcinoma cell lines, we investigated the concept of targeting and killing multidrug resistant cells in urogenital cancers. Renal carcinoma lines HTB44, 45, 46, and 47 expressed a relatively low, but detectable level of multidrug resistance (MDR)1 mRNA as indicated by Northern blot analysis, whereas prostate lines LNCaP and DU145 were found to be MDR1-negative. Anti-P-glycoprotein monoclonal antibody MRK16 was conjugated to Pseudomonas exotoxin (PE) by a stable thioether bond. Treatment with MRK16-PE resulted in a dose-dependent killing of multidrug resistant renal carcinoma cells, while non-MDR expressing prostate carcinoma cells were not affected. Addition of excess MRK16 blocked the effect of MRK16-PE. Furthermore, MOPC-PE, a non-MDR associated monoclonal antibody control conjugate, did not target and kill multidrug resistant renal carcinoma cells. Having established that MRK16-PE was active against and specific for multidrug resistant cells in culture, we also tested bioactivity in MDR-transgenic mice, whose bone marrow cells express the human MDR1 gene at a level approximately equal to that found in many human cancers. Again, MRK16-PE killed multidrug resistant bone marrow cells with high efficiency in an intact animal, and killing was blocked by unconjugated MRK16.

MeSH

Animal; Antibodies, Monoclonal; Bacterial Toxins; Carcinoma, Renal Cell; Drug Resistance; Exotoxins; Human; Immunotoxins; Kidney Neoplasms; Male; Mice; Mice, Transgenic; Prostatic Neoplasms; Support, Non-U.S. Gov't; Tumor Cells, Cultured

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theuer

Medline

Immunotoxins made with a recombinant form of Pseudomonas exotoxin A that do not require proteolysis for activity.

Theuer CP, Kreitman RJ, FitzGerald DJ, Pastan I

Cancer Res 1993 Jan 53:340-7

BROWSE : Cancer Res • Volume 53 • Issue 2

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Abstract

We used recombinant DNA technology to construct a mutant form of Pseudomonas exotoxin A (PE) called cysPE35 that contains amino acids 280-364 and 381-613 of PE. cysPE35 begins at the native PE proteolytic cleavage site and contains a single cysteine residue at position 287 that can be used to conjugate the toxin to monoclonal antibodies (MAbs). Unlike immunotoxins containing larger mutant forms of PE, such as PE40 or PE38, immunotoxins containing cysPE35 linked through a disulfide bond do not require proteolysis to generate a toxin fragment able to translocate to the cytosol. cysPE35 was conjugated to several MAbs and their activities were studied in vitro and in vivo. The concentration of toxin that inhibited protein synthesis as measured by a decrease in [3H]leucine incorporation of 50% of cysPE35 conjugated through a disulfide bond to the MAb HB21, which targets the human transferrin receptor, was 1 ng/ml on A431 cells. The MAb HB21 conjugated through a thioether bond to cysPE35 was much less active (concentration of toxin that inhibited protein synthesis as measured by a decrease in [3H]leucine incorporation of 50%, 200 ng/ml). An immunotoxin containing PE38 conjugated through either a disulfide or thioether bond to the MAb HB21 had a concentration of toxin that inhibited protein synthesis as measured by a decrease in [3H]leucine incorporation of 50% of 5 ng/ml, indicating that proteolysis of PE38 may be rate limiting in the action of these immunotoxins. Two other MAbs, LL2 and B3, were also conjugated through a disulfide bond to cysPE35. Both immunotoxins were also more active against cultured cells than conjugates using PE38 or PE40, and caused complete regression of human tumor xenografts growing in nude mice. In conclusion, we have constructed a mutant form of PE which must be coupled to MAbs through a disulfide bond to produce fully active immunotoxins that do not require proteolysis to generate a toxin fragment able to reach the cell cytosol.

MeSH

Animal; Antibodies, Neoplasm; Antigens, Tumor-Associated, Carbohydrate; Antineoplastic Agents; Base Sequence; Disulfides; Endopeptidases; Exotoxins; Female; Human; Immunotoxins; In Vitro; Mice; Mice, Nude; Molecular Sequence Data; Neoplasm Transplantation; Neoplasms, Experimental; Oligodeoxyribonucleotides; Receptors, Transferrin; Recombinant Proteins; Structure-Activity Relationship

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Recombinant F(ab') C242-*Pseudomonas* Exotoxin, but not the Whole Antibody-based Immunotoxin, Causes Regression of a Human Colorectal Tumor Xenograft[1]

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ABSTRACT

We have previously made a M_r 195,000 immunotoxin (IT) composed of mAb C242 coupled to *Pseudomonas* exotoxin A. This IT inhibited growth but did not cause regression of a human colorectal cancer xenograft growing in nude mice (W. Debinski *et al.*, J. Clin. Invest., *90:* 405–411, 1992). Since smaller proteins penetrate into tissues and tumors better than larger proteins, we have made a smaller recombinant (r) IT to overcome the hypothesized poor tumor penetration of the M_r 195,000 conjugate. This was accomplished by making a C242rF(ab')-based M_r 86,000 IT. To make rF(ab')-IT, the Fd and κ chains of mAb C242 were cloned, and κ was fused at the gene level to PE38QQR, a mutant form of *Pseudomonas* exotoxin. Both C242Fd and C242κ-PE38QQR were expressed in a bacterial expression system, and large amounts of the C242Fd attached via a disulfide bond to the C242κ-PE38QQR were obtained. The C242rF(ab')-IT covalently linked heterodimer has a 50% inhibitory concentration of 0.2–2.0 ng/ml (2–20 pM, respectively) on human colon adenocarcinoma cell lines that express the C242 antigen. When injected into mice bearing Colo205 tumors, the C242rF(ab')-PE38QQR caused an immediate regression of the tumors, while the C242-PE38QQR conjugate had only a growth inhibitory effect. In addition, several cures were obtained. Our results indicate that rIT C242F(ab')-PE38QQR is a much more potent antitumor agent than an IgG conjugate.

INTRODUCTION

mAbs coupled to toxins, drugs, or isotopes represent a promising modality for the treatment of hemopoietic and epithelial cancers (1, 2). mAbs linked to toxins, named ITs,[3] have been shown to have activity in clinical trials involving patients with lymphomas and leukemias, in purging of the autologous bone marrow transplants and in animal models of human solid tumors (1–5). Several mAbs have been identified that react with tumor-associated antigen and have a relatively low level of cross-reactivity with normal tissues (6, 7).

We have previously made ITs composed of mAb C242 and PE or its derivatives (7). We have demonstrated definite antitumor effects of the ITs, but they could not produce regression of a human colorectal cancer growing in athymic mice (7). The C242 antigen is present on the majority of human colorectal cancers (7). This antigen is a sialylated carbohydrate, and it is attached to a protein core (8). Since we excluded the possibility of a loss of the C242 antigen by Colo205 cells producing tumors in nude mice, and because we also excluded neutralization of the IT by the C242 shedding, we hypothesized that the incomplete antitumor effect is probably due to inefficient tumor penetration by the high molecular weight mAb C242-IT (7).

PE is a bacterial toxin made up of three major domains (9, 10). Domain Ia of PE binds to its cell surface receptor, domain II catalyzes the translocation of the toxin into the cytosol, and domain III contains the ADP ribosylation activity that inactivates elongation factor 2 and leads to cell death (reviewed in Refs. 1 and 3; Fig. 1). To kill a cell, PE must be cleaved by an intracellular protease between arginine 279 and glycine 280 to produce a M_r 37,000 C-terminal fragment (Fig. 1). This M_r 37,000 fragment, composed of all of domain III and a portion of domain II, is capable of penetrating the cytosol. Several mutants of PE have been used to make ITs or chimeric toxins (1, 3).

Recent advances in genetic engineering combined with the knowledge of the tertiary structure of immunoglobulins have enabled one to design rITs in which the specificities of antibody-combining sites are retained. The antibodies are produced as $F(ab')_2$s or F(ab')s, or as SC (Fv) fragments (11–17). The latter are composed of the variable heavy and light chains connected together by a polypeptide linker. One of the benefits of making recombinant truncated antibodies is that recombinant techniques offer a convenient and inexpensive way to prepare antigen-binding regions of mAbs. In addition, $F(ab')_2$s and F(ab')s cannot be made from some antibodies using proteolytic methods; mAb C242 is such an example.

There is evidence to show that lower molecular weight forms of ITs penetrate tumors better and show better antitumor activities (18–21). Since antitumor experiments with the chemical conjugates of mAb C242 and mutant forms of PE suggested that the ITs did not penetrate effectively into the Colo205 xenografts (7), we have now produced a smaller rIT with spec-

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[3] The abbreviations used are: IT, immunotoxin; PE, *Pseudomonas* exotoxin; rIT, recombinant IT; hIL4, human interleukin 4; hIL13, human interleukin 13; IC_{50}, 50% inhibitory concentration; rF(ab'), recombinant F(ab'); SCIT, single-drain immunotoxin.

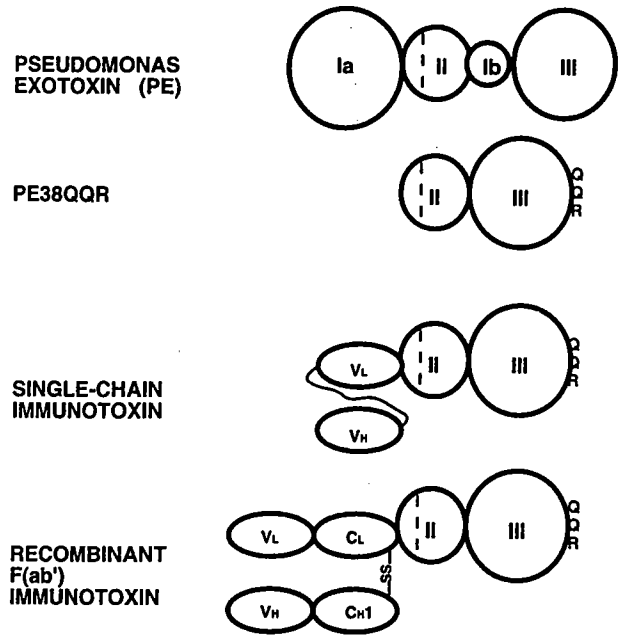

Fig. 1 Schematic drawing of multidomain proteins: PE, its derivative, PE38QQR, SCIT, and heterodimeric rF(ab')-IT. *Circles* correspond to the structural domains of PE: domain Ia (amino acids 1–252) is a binding domain, domain II (amino acids 253–364) is a place of the proteolytic cleavage (*interrupted line*), domain Ib (amino acids 365–404) has no known function, and domain III (amino acids 405–613) is the ADP-ribosylating domain. PE38QQR: domain Ia and amino acids 365–380 in Ib are deleted, plus the three lysine residues in domain III at positions 590, 606, and 613 are changed to two glutamines and arginine (*QQR*) (20, 27). *Ovals*, variable heavy (V_H), variable light (V_L), and constant regions of the light chain (C_L) and the heavy chain (C_H1). *SS*, disulfide bond. *Continuous line*, a flexible polypeptide linker composed of 3 × [(4 × Gly) Ser]. The intradomain disulfide bonds are not drawn.

ificity toward the C242 antigen. We describe here a rIT in which the κ chain of mAb C242 IgG1 is fused to a truncated mutant form of PE, PE38QQR. The Fd fragment is attached to this fusion by a disulfide bond using the last C-terminal cysteine residue of the κ chain, as in natural IgG (Fig. 1). Using this approach, the rF(ab')-IT preserves the specificity of mAb C242 and is a very potent IT. With this form of mAb C242-IT, we have observed tumor regressions and several cures in the Colo205 model of human colon cancer for the first time.

MATERIALS AND METHODS

Restriction endonucleases and DNA ligase were obtained from New England Biolabs (Beverly, MA), Bethesda Research Laboratories (BRL, Gaithersburg, MD), and Boehringer Mannheim (Indianapolis, IN). [^{3}H]leucine and ^{125}I were purchased from Amersham Corporation. Fast protein liquid chromatography columns and media were purchased from Pharmacia (Piscataway, NJ). Oligonucleotide primers were synthesized either at Applied Biosystem oligonucleotide synthesizer (National Cancer Institute/NIH, Bethesda, MD) or at Pharmacia's Gene Assembler (Research Centre, Hôtel-Dieu Hospital, Montreal, Quebec, Canada). A PCR kit was obtained from Perkin Elmer/Cetus (Norwalk, CT). Random primers, RNase, dNTPs, BSA, and Moloney murine leukemia virus reverse transcriptase were obtained from Pharmacia. hIL4 and hIL13 were made by W. D.

Plasmids, Bacterial Strains, and Cell Lines

Plasmid pSG242-κPE38, which encodes C242κ-PE38QQR, and plasmid pSGFdN1, which encodes C242Fd recombinant protein, each carries a T7 bacteriophage late promoter, a T7 transcription terminator at the end of the open reading frame of the protein, and an f1 origin of replication and gene for ampicillin resistance (7).

Recombinant proteins were expressed in *Escherichia coli* BL21 (λDE3) under control of the T7 late promoter (22). Plasmids were amplified in *E. coli* (HB101 or DH5α high-efficiency transformation; BRL), and DNA was extracted using Qiagen kits (Chatsworth, CA).

Cloning the Genes of mAb C242

cDNA Synthesis of the mAb C242 Variable Chains. This was performed essentially as described (12). Total RNA was used for the first-strand cDNA synthesis using random primers. The correct V_L was PCR cloned on information on the amino acid sequence of the NH_2 terminus of the mAb C242 light chain. To clone the heavy chain, which is *N*-terminally blocked, the universal primers annealing to the conserved regions among murine antibodies were used. The V_L and V_H were extracted, separated from the excess of primers, and digested with appropriate restriction endonucleases for subcloning into the vectors carrying various forms of PE. The details of all of these procedures are available from the authors upon request. Plasmid pWD2424 encodes C242(Fv)PE38QQR in which the COOH terminus of the V_H is extended by a triple repeat of [(4 × Gly) Ser] and fused to the NH_2 terminus of the V_L, which in turn is fused to PE38QQR (Fig. 1; Single-chain immunotoxin).

Cloning the Genes Encoding the Whole mAb C242. The cDNAs for the heavy and light chains were obtained by screening a cDNA library with probes for IgG1 and κ constant regions. The cDNA synthesis was done with the UniZAP XR cDNA synthesis kit (Stratagene). Both light and heavy chains were cloned into pBluescript SK(−) *Eco*RI/*Xho*I sites (Stratagene). Plasmid pKGE761 encodes the light chain, and plasmid pKGE762 encodes the heavy chain of C242.

Construction of Plasmids Encoding C242rF(ab')-IT

The chimeric gene encoding C242κ fused to PE38QQR (plasmid pSG242-κPE38) was constructed by first amplifying κ chain using PCR. The primers used for κ chain amplification contained sites for restriction endonucleases *Nde*I and *Hin*dIII at the 5′ and 3′ end of the C242κ cDNA, respectively. The 689-bp DNA fragment was obtained after 25 cycles of PCR using pKGE761 as a template and digested with *Nde*I and *Hin*dIII to give a 665-bp fragment. This fragment was ligated to the vector obtained from plasmid pWDMH4QQR, encoding hIL4-PE38QQR (23), cut with *Nde*I and *Hin*dIII to make plasmid pSG242-κPE38. Plasmid pSG242-κPE38QQRD^{553} (with Glu^{553} of PE deleted) was constructed by cleaving plasmid pSG242-κPE38 with *Bam*HI and *Eco*RI, and replacing a 460-bp

fragment with a similarly cleaved fragment from pWDMH4D encoding hIL4-PE4ED^{553} (24).

The gene encoding C242Fd was also constructed first by PCR amplification of the Fd chain. The primers contained sites for restriction endonucleases *Nde*I and *Sst*I. The 687-bp DNA fragment was obtained after 25 cycles of PCR using pKGE762 as a template and digested with *Nde*I and *Sst*I to give a 663-bp fragment. This fragment was ligated to the vector obtained from plasmid pWD2425, encoding C242(Fv)PE38KDEL,[4] cut with *Nde*I and *Sst*I to make plasmid pSG242-Fd. Therefore, the cloned Fd chain has two additional amino acids at its C-terminal end: glutamic acid and leucine. However, the expression of C242Fd was very poor in *E. coli*. Since we observed a good expression of the C242(Fv)PE38QQR (plasmid pWD2424) and the N-terminal ends of the Fd (pSG242-Fd) and C242(Fv)PE38QQR differ at several N-terminal amino acids in the constant region, we replaced these amino acids in pSG242-Fd with the ones from pWD2424. This was done by digesting both plasmids with the *Nde*I enzyme (there is an *Nde*I site in the middle of the C242V_H) and ligating a 177-bp fragment obtained from pWD2424 into a *Nde*I-cut pSG242-Fd, to produce plasmid pSG242-FdN1.

Several plasmids were sequenced using the dideoxynucleosides termination method with the USB (Cleveland, Ohio) kit Sequenase Version 2.0.

Expression and Purification of Recombinant Proteins

E. coli BL21 (λDE3) cells were transformed with plasmids of interest, cultured in 1.0 liter Super broth with 100 μg ampicillin, 4 g glucose, and 0.4 g $MgSO_4$/1.0 liter culture. The cells were grown up to A_{650} = 1.0–2.5 and induced with isopropyl-β-D-thiogalactoside for 90 min. The separation of different cellular fractions was performed as described previously (24), and both C242Fd and C242κ-PE38QQR were localized to the inclusion bodies. The procedure for isolation of the fragmented chains of C242 from the inclusion bodies was also described previously (24, 25). Briefly, the spheroplasts were lysed with lysozyme and the inclusion bodies were washed with Triton X-100 followed by extensive washing with 50 mM Tris-HCl (pH 7.4)/20 mM EDTA. The proteins were dissolved in 6 M guanidine solution, and renaturation was carried out in a dithioerythritol and oxidized glutathione reduction-oxidation mixture. Twice as much of the C242Fd than C242κ-PE38QQR, on a molar basis, was added to the same renaturation solution. After dialysis against 20 mM Tris-HCl (pH 7.4)/5 mM EDTA, the monomer of the renatured heterodimeric protein was first purified by chromatography on Q-Sepharose Fast Flow (HR 16/10 column). The renatured protein was eluted from Q-Sepharose with 0.28 M NaCl, and early fractions contained a predominantly monomeric form of the rF(ab')-IT dimer. Later eluates contained mainly high molecular weight multimers and had very limited cytotoxic activities.[4] Fractions containing predominantly the monomeric form of the rF(ab')-IT were pooled, concentrated, and injected onto a size exclusion chromatography column (Sephacryl S-200, HR 16/50).

The SCITs were extracted from *E. coli* and purified according to the method described above, and also under the conditions of the previously decribed procedure (26).

Protein concentration was determined according to the Bradford assay (Pierce "Plus," Rockford, IL) using BSA and IgG as standards.

The chemical conjugates of mAb C242 and PE mutants were prepared according to the previously described procedure (7).

Protein Synthesis Inhibition Assay

The cytotoxic activity of ITs was tested on cancer cell lines, such as Colo205 and Colo201 colon carcinomas, CRL 1739 gastric carcinoma, A431 epidermoid carcinoma, and also on normal murine L929 fibroblasts. The cells were maintained under conditions recommended by the American Type Culture Collection. Usually 1 × 10^4 cells/well were plated in a 24-well tissue culture plate in 1 ml medium, and various concentrations of ITs were added 20–28 h after cell plating. ITs were diluted in 0.1% BSA/PBS, and 25 μl of each dilution were added to 1 ml of cell culture medium. After a 20-h incubation with ITs, [^{3}H]leucine was added to the cells for 3 to 5 h, and the cell-associated radioactivity was measured using a beta counter. For blocking studies (*a*) mAb C242 alone; (*b*) HB21 (IgG1κ), an irrelevant mAb reacting with a human transferrin receptor; or (*c*) hIL13 or hIL4, irrelevant to antibodies recombinant proteins, was added to cells for 20 to 30 min before the addition of ITs. Data were obtained from the average of duplicates, and the assays were repeated several times.

Competitive Binding Assay

We determined the binding abilities of C242-rITs as compared to the antibody alone. To this end, we labeled C242 with ^{125}I using a lactoperoxidase method exactly as described (7). C242 (50 μg) was mixed with 1.0 mCi of the isotope. Labeled antibody was separated from unreacted isotope on a PD10 column. The fractions collected from gel filtration were enriched in BSA to a 0.1% (w/v) final concentration. Binding assays were done using saturation and displacement analyses. Briefly, Colo205 cells were seeded at 0.5 × 10^5 cells/well in 24-well tissue culture plates 48–72 h before the experiment. The plates were placed on ice, and cells were washed with ice-cold PBS without Ca^{2+}/Mg^{2+} in 0.1% BSA. Then, increasing concentrations of C242 or C242 ITs were added to cells and incubated for 30 min before the addition of a fixed amount of ^{125}I-labeled C242 for 1.5 to 2 h. After incubation, the cells were washed, lysed with 0.1 N NaOH, and the γ radioactivity was counted.

Nude Mouse Model of a Human Colorectal Cancer

Colo205 cells (3 × 10^6 cells/mouse in 100 μl Ca^{2+}/Mg^{2+}-free PBS) were injected s.c. into female nude mice on day 0. Each experimental group consisted of four to five animals. Tumors developed in all injected animals, and the tumor size reached at least 5 × 4 mm on days 7–8. The mice started to receive ITs i.p. (100 μl, injection volume; 0.1% BSA/PBS, vehicle) on day 7 or day 8. Tumors were measured with a caliper, and the formula for tumor volume calculation was as previously reported (7).

[4] W. Debinski *et al.*, unpublished observations.

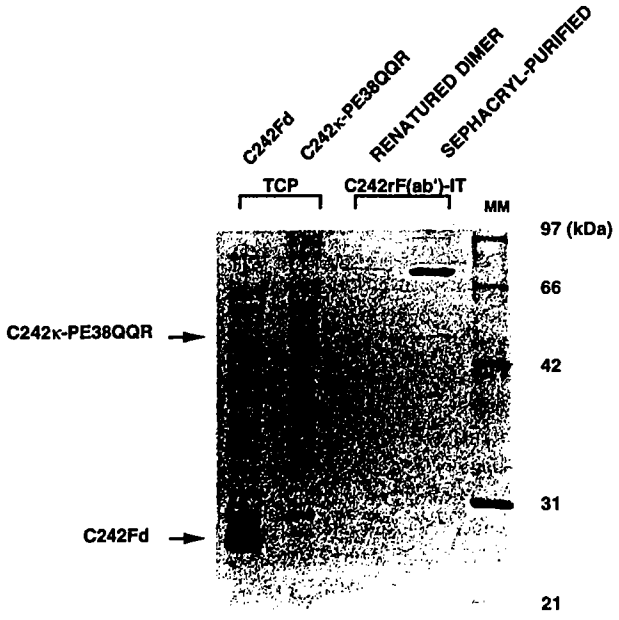

Fig. 2 Localization in *E. coli* of the C242Fd and C242κ-PE38QQR and purification of C242rF(ab')-PE38QQR. Fifteen percent SDS-PAGE stained with Coomassie blue. Total cell pellet (*TCP*) of transformed BL21(λDE3) bacteria (2 μl/lane of 75 ml suspension in sucrose from 1.0 liter culture). The dimer of C242rF(ab')-IT was reconstituted under reduction-oxidation conditions (25 μl/lane of 1000 ml renaturation solution). Sephacryl S-200-purified protein was loaded at 10 μg/lane.

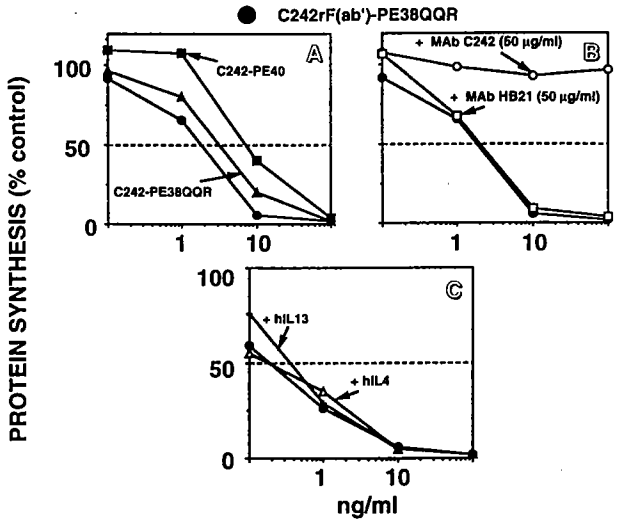

Fig. 3 *A*, cytotoxicities of the chemical conjugates: C242-PE40 and C242-PE38QQR *versus* recombinant monovalent C242rF(ab')-PE38QQR on Colo205 cells. *B*, inhibition of the cytotoxicity of C242rF(ab')-PE38QQR on Colo205 cancer cell line by mAb C242 and an irrelevant mAb HB21. Both C242 and HB21 were added at a concentration of 50 μg/ml. *C*, inhibition of the cytotoxicity of C242rF(ab')-PE38QQR on Colo201 cells by interleukins 4 and 13 (1 μg/ml). - - -, 50% of [^{3}H]leucine incorporation.

RESULTS

Expression of the rF(ab')-IT. A plasmid was constructed in which a gene encoding C242κ was fused to a gene encoding a mutant form of PE, PE38QQR. In the latter, domain Ia of PE and a portion of domain Ib (amino acids 365–380) are deleted, and the amino acids at positions 590, 606, and 613 are changed to two glutamines and an arginine, respectively. These mutations in PE abolish the binding of the toxin to its receptor, eliminate one disulfide bridge-bonded loop within domain Ib, and eliminate all of the lysines from domain III of PE (Fig. 1; Refs. 20 and 27). Another plasmid encodes the Fd fragment of C242 with two additional amino acids at its C-terminal end (Glu and Lys). As illustrated in Fig. 1, the heterodimeric protein is composed of the C242Fd fragment (212 amino acids), and the C242κ-PE38QQR fragment (559 amino acids), of which 212 amino acids are from C242κ and 347 amino acids are from PE.

C242κ-PE38QQR, encoded by plasmid pSG242-κPE38, and C242Fd, encoded by plasmid pSG242FdN1, were expressed individually in *E. coli* BL21 (λDE3) under the control of a bacteriophage T7 late promoter. The recombinant proteins were overexpressed in *E. coli* as seen by using SDS-PAGE analysis of the total cell extract (Fig. 2). We found that neither protein was secreted into the periplasm; they were present within the spheroplasts and confined to the inclusion bodies.[4] To prepare rIT in a heterodimeric form, the inclusion bodies were washed and dissolved in 6 M guanidine, and the two components were combined into one tube and subjected to renaturation (Fig. 2). A heterodimer was formed during renaturation of the mixture of C242κ-PE38QQR and C242Fd. Purification of the renatured recombinant toxin was carried out on successive Q-Sepharose and Sephacryl columns. C242rF(ab')-PE38QQR appeared as a single entity on a sizing column and was eluted in fractions expected for a protein with a M_r 86,000 (Fig. 2). Almost 25% of the proteins contained within the inclusion bodies were recovered in a monomeric form.

Cytotoxicity of the C242rF(ab')-IT. We first determined the cytotoxic potency of a monomer of the C242rF(ab')-PE38QQR obtained from the Sephacryl column. Although a majority of human colorectal cancer samples stain for the C242 antigen (7), only a few of the established cell lines express this antigen. Among them are the Colo205 and Colo201 human colorectal cancer cell lines which express the antigen homogenously, and the HT29 cells which express the antigen heterogenously on about one-half of the cells[4] (7). This limits the number of cell lines that can be used for verification of the activity of C242rF(ab')-PE38QQR.

In the Colo201 and Colo205 cell lines, protein synthesis was inhibited by 50% (IC_{50}) at 0.2 and 2.0 ng/ml (2.3 pM and 23 pM, respectively) of C242rF(ab')-PE38QQR (see also data in Figs. 3 and 4). This very potent cytotoxic effect prompted us to evaluate further the rF(ab') IT molecule. We next compared the cytotoxic activity of C242rF(ab')-PE38QQR to that exhibited by the chemical conjugate with IgG C242-PE40, the first active form of C242-IT described (7). C242-PE40 shows an activity (IC_{50}) of 8 ng/ml (41 pM) on Colo205 cells (Fig. 3*A*). We have previously found that ITs containing the PE38QQR mutant of PE, instead of PE40 (or PE38), were more cytotoxic (27). This is due to the production of more homogenous ITs, which all should be active and kill cells (27). Therefore, we also prepared a chemical conjugate of mAb C242 with PE38QQR. C242-

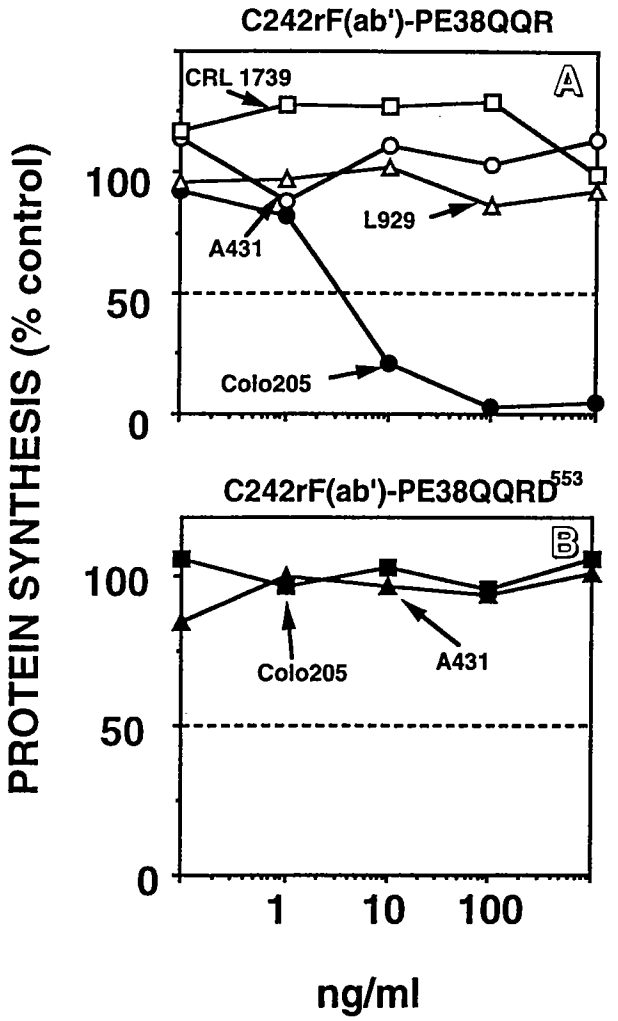

Fig. 4 Inhibition of protein synthesis in different cell lines by C242rF(ab')-PE38QQR (*A*) and C242rF(ab')-PE38QQRD^{553} (*B*), its toxin-inactive mutant. - - -, 50% of [^{3}H]leucine incorporation.

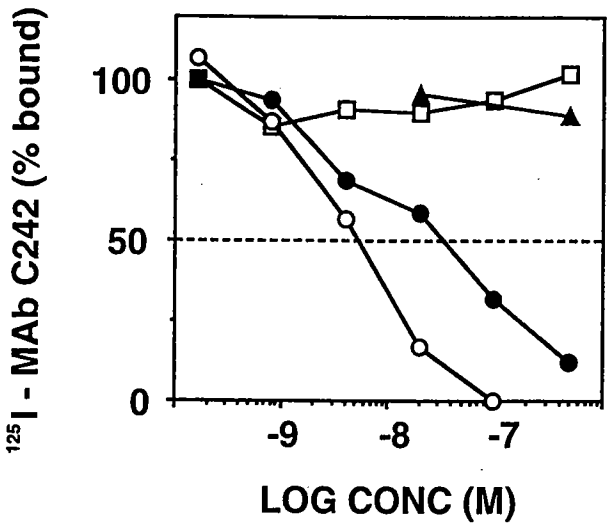

Fig. 5 Competitive binding assay on Colo205 colorectal cancer cells. Data are expressed as a percentage of total ^{125}I-labeled C242 binding to cells. The points are the average of two determinations and are representative of three experiments. - - -, 50% of ^{125}I-labeled C242 displacement. ○, mAb C242; □, mAb HB21; ●, C242rF(ab')-PE38QQR; ▲, C242(Fv)PE38QQR.

PE38QQR was more active (IC_{50} = 3.5 ng/ml; 15 pM) than C242-PE40, as expected. The recombinant monovalent form of C242-IT, C242rF(ab')-PE38QQR, was the most active among the three studied ITs (Fig. 3*A*) with a IC_{50} of 1 ng/ml (11 pM) on Colo205 cells. Thus, we have succeeded in constructing a recombinant C242-based IT in which cytotoxic activity is, on a molar basis or on a valency basis, equal or superior to that of chemical conjugates.

Specificity of the C242rF(ab')-IT Cytotoxic Action. Several lines of evidence show that the cytotoxic effect of C242rF(ab')-PE38QQR is specific for the C242 antigen. First, the activity of C242rF(ab')-PE38QQR was prevented by an excess of native C242 IgG. Fig. 3*B* shows a typical experiment in which Colo205 cells were treated with C242rF(ab')-PE38QQR with and without mAb C242. A 50-fold excess of mAb C242 (87-fold molar excess) blocked the action of C242rF(ab')-PE38QQR completely. Further evidence for the specificity of the cytotoxic action of C242rF(ab')-PE38QQR is provided by the lack of an inhibitory effect of mAb HB21, an isotype-matched control (Fig. 3*B*). Moreover, a 714-fold molar excess of recombinant hIL4 or recombinant hIL13 did not prevent the cytotoxic action of C242rF(ab')-PE38QQR (at a concentration of 1 ng/ml of the toxin) on another colon cancer cell line, Colo201 (Fig. 3*C*). The Colo201 cell line expresses receptors for the two cytokines.[4]

For the successful immunotherapy of cancer, it is important that an IT is cytotoxic to targeted cells with little or no toxicity to cells not expressing the C242 antigen. Therefore, we tested C242rF(ab')-PE38QQR on cells without the C242 antigen and found it was without any cytotoxicity (up to 1 μg/ml; 11.6 nM) on CRL1739, A431, and L929 cells (Fig. 4*A*). These results provide further evidence that the recombinant C242-IT kills only cells that express the C242 antigen. A rIT with no ADP ribosylation activity, C242rF(ab')-PE38QQRD^{553}, was also prepared and was not cytotoxic to Colo205 cells or to the C242 antigen-negative A431 cells (Fig. 4*B*).

Binding Ability of the Monovalent mAb C242-IT. To determine the affinity of C242rF(ab')-PE38QQR for target antigen, we performed competitive binding assays using ^{125}I-labeled C242. As shown in Fig. 5, C242rF(ab')-PE38QQR competes for the binding of ^{125}I-labeled C242 to Colo205 cells with an apparent IC_{50} of 32 nM. The same result was obtained with the C242rF(ab')-PE38QQRD^{553} mutant.[4] An irrelevant isotype-matched antibody (HB21) did not have any effect on the binding of ^{125}I-labeled C242 to target cells (Fig. 5). Similar results were obtained on Colo201 cells.[4] Since the affinity of C242 is 6 nM (Fig. 6), the monovalent rF(ab')-IT binds about 20% as well on a molar basis, and about 40% on a valency basis, as divalent IgG C242. However, it binds the antigen in a manner similar to the chemical conjugates of PE with the antibody (see data in Ref. 7).

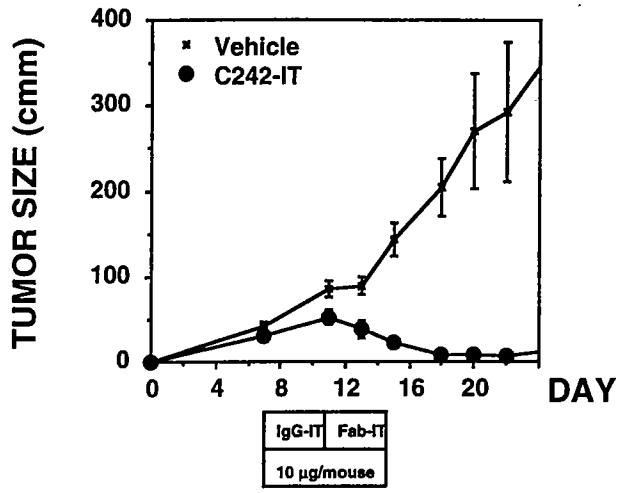

Fig. 6 Antitumor effect of a divalent C242 IgG-IT and a monovalent C242rF(ab')-IT on Colo205 xenografts in nude mice. Colo205 cells were inoculated s.c. on day 0 (3×10^6 cells/mouse), and the mice were treated first with the IgG-IT followed by the rF(ab')-IT i.p. on the days *underlined by the rectangles. Vertical bars*, SEs of individual points.

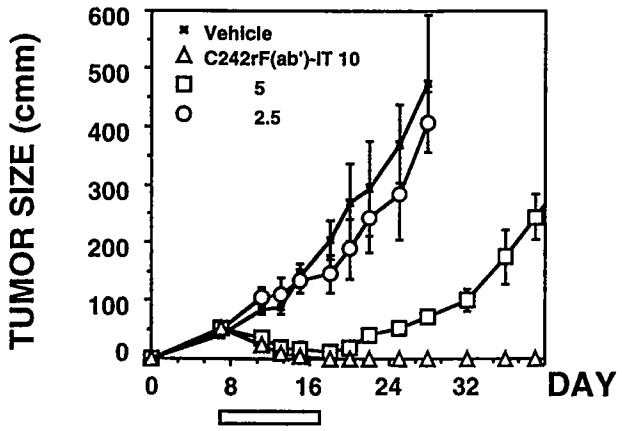

Fig. 7 Dose dependence of the C242rF(ab')-PE38QQR antitumor action; 2.5, 5, and 10 correspond to µg/mouse/day. □, period of daily i.p. injections of the C242rF(ab')-IT. *Vertical bars*, SEs of individual points.

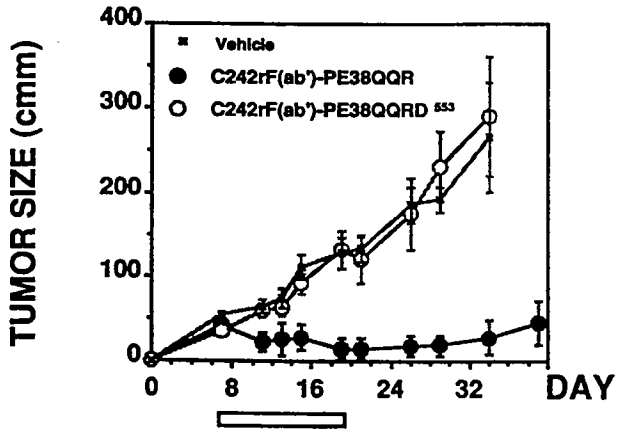

Fig. 8 Cure of Colo205 tumors produced by every other day injections of C242rF(ab')-PE38QQR (10 µg/mouse/day). C242rF(ab')-PE38QQRD[553] was given in the same schedule as the active rIT. □, days treatment was carried out. *Vertical bars*, SEs of individual points.

Antitumor Activity of the C242rF(ab')-IT. Based on the results presented above, we conclude that C242rF(ab')-PE38QQR is a desirable agent for an *in vivo* study with tumor-bearing mice. This is because we found only a small difference in the *in vitro* cytotoxic potency between the rF(ab')-IT and the equivalent IgG chemical conjugate. The latter has never caused any significant regression of a human colorectal tumor in athymic mice. Neither have PE conjugates with mAb HB21, which target human transferrin receptors in Colo205 model[4] (7). Conjugates of mAb C242 with ricin A chain have not been able to regress Colo205 tumors either (28). It was thus of interest to test whether the smaller rC242-IT could be more effective.

Nude mice were implanted with 3×10^6 Colo205 cells s.c. on day 0, and on day 7 small established solid tumors (5×4 mm) appeared in all of the mice (Fig. 6). On that day, the mice started to receive i.p. either 10 µg/mouse/day of the C242-PE38QQR conjugate for 5 days or the vehicle. The conjugate inhibited the growth of Colo205 tumors when compared to the vehicle treatment (Fig. 6). On day 11, when tumors were 5×5 mm in diameter in the IT-treated group, we exchanged the conjugate for C242rF(ab')-PE38QQR; the rIT was also administered at a 10 µg/mouse/day for 5 days. We observed an immediate and dramatic decrease in tumor size in response to the treatment with the monovalent rIT; even so the tumor size was bigger than at the commencement of treatment with the conjugate (Fig. 6). At the end of the treatment, and at least for the succeeding 10 days, the mice were bearing very small tumors. Around day 25, the tumors started to regrow but at a slower rate of growth than that observed in the vehicle groups.[4] Thus, the first animal experiment demonstrated that the monovalent rIT can do what the divalent conjugate could not; *i.e*, it caused regression of the colorectal cancer.

We also tested whether the antitumor effect of C242rF(ab')-PE38QQR is dose dependent. Thus, the mice were treated, starting on day 8, with (*a*) 2.5 µg, (*b*) 5 µg, or (*c*) 10 µg/mouse/day of the rIT for 9 days, or (*d*) the vehicle (Fig. 7). At the lowest dose, the rIT had little effect on Colo205 tumor growth. However, at 5 µg/day, the tumors regressed significantly for about 10 days (Fig. 7). The highest dose of C242rF(ab')-PE38QQR caused complete regression in all mice, but at the total dose of 90 µg/mouse (daily injections) was very toxic; only one tumor-free mouse survived the protocol.

To optimize the therapeutic response to C242rF(ab')-PE38QQR, we administered the rIT at 10 µg/mouse every other or every third day for a total of six injections. We found that under this schedule there were no deaths or weight loss. Importantly, in mice receiving injections every second day, the tumors regressed in the course of treatment; 60% of the mice had unmeasurable colorectal tumors till day 40 (Fig. 8). The rIT with no ADP ribosylation activity, C242rF(ab')-PE38QQRD[553], had no effect on tumor growth (Fig. 8).

DISCUSSION

We have made a monovalent rIT composed of C242rF(ab') and a PE derivative PE38QQR. C242rF(ab')-PE38QQR exerts potent cytotoxic activity on cancer cell lines expressing the C242 antigen which, on a weight or valency basis, is better than or equal to that of the two chemical conjugates C242-PE40 and C242-PE38QQR, respectively. We observed an excellent antitumor effect of C242rF(ab')-PE38QQR on a human colorectal cancer xenograft. In fact, this is the first report describing the regression of Colo205 tumors in response to immunotherapy.

Specificity of C242rF(ab')-PE38QQR Action. C242rF(ab')-PE38QQR is a very cytotoxic agent with IC_{50}s ranging from 0.2 to 2 ng/ml (2.3–23 pM) on the C242 antigen-positive cell lines used in our study. The cytotoxic activity was blocked by an excess of C242, but not of HB21, indicating that the action of C242rF(ab')-PE38QQR is specific. C242rF(ab')-PE38QQR has no activity on cells not expressing the C242 antigen. In another test of specificity, we found that a mutant toxin with no ADP ribosylation activity, C242rF(ab')-PE38QQRD^{553}, had no significant impact on protein synthesis in target cells at a concentration of <1 μg/ml (11.6 nM). However, it binds the C242 antigen in a manner similar to the active IT. Thus, only the active rIT is responsible for killing a subset of specific cells.

C242rF(ab')-IT, Unlike the Whole Antibody Conjugate, Is Capable of Causing Regression of Colorectal Tumors. The monovalent recombinant form of C242-IT exhibited a remarkable antitumor effect, much better than that of the IgG conjugates. Also, previous efforts using the antibody-dependent macrophage-mediated cytotoxicity approach to treat established Colo205 tumors was not successful (29). Using successive treatments of the same animals, we found that the divalent C242-PE38QQR slowed the growth of Colo205 tumors, whereas the monovalent C242rF(ab')-PE38QQR quickly regressed the IgG conjugate-treated tumors. The antitumor action of C242rF(ab')-PE38QQR is dose dependent. At the 10-μg/day dose, injections of C242rF(ab')-PE38QQR every other day are well tolerated, and with this alternate regimen we have seen several permanent regressions. By making a monovalent rIT of considerably smaller size than the whole antibody conjugate, we have achieved our goal of efficient treatment of small established solid tumors in a nude mouse model of human colorectal cancer.

Monovalent *versus* Divalent ITs. Our current data confirm that rF(ab')-IT binds relatively well to the targeted antigen. Another IT exhibited the same properties (30), and the recombinant F(ab') fragments themselves seem not to loose their binding abilities when compared to unfragmented antibodies (15–17). Also, the F(ab')-IT, in which the F(ab') fragment had been obtained using proteolytic digestion and coupled to the toxin at a defined site, had both specific binding toward the antigen and cytotoxic activity preserved (20). These observations should be useful for further development of antibody-based therapeutics.

Unnatural Extension of the Light Chain by a Non-IgG Protein Moiety in a rF(ab') Does Not Hamper a Proper Reconstitution of the C242 Antibody Combining Site. In native IgG, the Fd portion is extended by a hinge region and subsequent constant C_H2 and C_H3 regions; the light chain ends at the C_L1 with a cysteine residue that forms an interchain disulfide bridge with the Fd. In the rF(ab')-IT, the Fd terminates two amino acids after the last cysteine residue upstream of the hinge region, and the κ chain merges with the toxin; the toxin is located just after the cysteine that forms the interchain disulfide bridge without introducing a connecting linker (Fig. 1). Such rearrangements have been shown to allow a proper reconstitution of the antibody combining site in another mAb besides C242 (30). Interestingly, this was also associated with a good recovery of the monomer of the recombinant heterodimeric protein.

High Affinity Combining Site of mAb C242 Can Be Reconstituted in a rF(ab')-IT but not in a (*a*) Proteolysed Fragment, or (*b*) Single-Chain (Fv) IT. We successfully reconstituted the C242 antibody combining site in the C242rF(ab')-PE38QQR molecule. We were unsuccessful in producing $F(ab')_2$ fragments by proteolysis using a variety of techniques (31). We also prepared a SCIT with mAb C242 in which the Fv fragment containing the variable heavy and light chains of mAb C242 was connected by a polypeptide linker and fused to a recombinant form of PE (Fig. 1). However, this SCIT has a very low affinity for C242 antigen-bearing Colo205 cells, as seen in a competitive binding assay (Fig. 5). It also has a much lower cytotoxicity than the chemical conjugate. There may be several reasons for the low binding and activity of the SCIT. First, the linker connecting the variable chains may interfere with the antibody combining site (32). Second, proper refolding of C242(Fv)PE38QQR may require special renaturation conditions which were not identified. Third, for some IgGs it may be important to have the first constant regions present for appropriate refolding and/or support of the variable regions.

C242rF(ab')-PE38QQR is a recombinant monovalent IT in which the light chain is fused with the toxin and covalently linked to the Fd by a disulfide bridge. This IT is a potent cytotoxic agent for cancer cells expressing the C242 antigen and can be easily obtained in large amounts. C242rF(ab')-PE38QQR has excellent antitumor activity on a human colorectal cancer xenograft and merits further preclinical evaluation.

ACKNOWLEDGMENTS

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Kreitman

Medline

Properties of chimeric toxins with two recognition domains: interleukin 6 and transforming growth factor alpha at different locations in Pseudomonas exotoxin.

Kreitman RJ, Siegall CB, Chaudhary VK, FitzGerald DJ, Pastan I

Bioconjug Chem 3:63-8

BROWSE : Bioconjug Chem • Volume 3 • Issue 1

VIEW : **MEDLINE**, full MEDLINE, related records

Abstract

Pseudomonas exotoxin (PE) is a potent cytotoxic agent that is composed of 613 amino acids arranged into three major domains. We have previously identified two positions where ligands can successfully be placed in PE to direct it to cells with specific surface receptors. One site is at the amino terminus and the other is close to but not at the C-terminus. To examine the possibility of constructing oncotoxins with two different recognition elements that will bind to two different receptors, we have placed cDNAs encoding either transforming growth factor alpha (TGF alpha) or interleukin 6 (IL6) at the 5' end of a PE gene and also inserted a cDNA encoding TGF alpha near the 3' end of the PE gene. The plasmids encoding these chimeric toxins were expressed in Escherichia coli and the chimeric proteins purified to near homogeneity. In all the new toxins, the TGF alpha near the C-terminus was inserted after amino acid 607 of PE and followed by amino acids 604-613 so that the correct PE C-terminus (REDLK) was preserved. For each chimera, the toxin portion was either PE4E, in which the cell binding domain (domain Ia) is mutated, PE40, in which domain Ia is deleted, or PE38, in which domain Ia and part of domain Ib are deleted. These derivatives of PE do not bind to the PE receptor and allow 607, 355, or 339 amino acids, respectively, between the two ligands.(ABSTRACT TRUNCATED AT 250 WORDS)

MeSH

Animal; Binding Sites; Escherichia coli; Exotoxins; Immunotoxins; Interleukin-6; Lethal Dose 50; Mice; Mice, Inbred BALB C; Plasmids; Receptor, Epidermal Growth Factor; Transforming Growth Factor alpha; Tumor Cells, Cultured

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Order Document

Gawlak

Medline

Basic fibroblast growth factor-Pseudomonas exotoxin chimeric proteins; comparison with acidic fibroblast growth factor-Pseudomonas exotoxin.

Gawlak SL, Pastan I, Siegall CB

Bioconjug Chem 4:483-9

BROWSE : Bioconjug Chem • Volume 4 • Issue 6

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Abstract

We have constructed growth factor-toxin chimeric molecules composed of basic fibroblast growth factor (bFGF) and two different binding mutant forms of Pseudomonas exotoxin termed bFGF-PE40 and bFGF-PE4E KDEL. The chimeric molecules were expressed in Escherichia coli and localized to both inclusion bodies and the spheroplast cytoplasm. The bFGF-toxin fusion protein that was isolated and purified from inclusion bodies was 3-fold more active in inhibiting protein synthesis than that purified from spheroplast cytoplasm. Immunoreactivity of purified bFGF-toxin fusion protein to anti-bFGF antibodies was similar to that of native bFGF, as determined by ELISA analysis. A variety of carcinoma cell lines were sensitive to bFGF-PE40 and bFGF-PE4E KDEL, including H3396 (breast), Hep G2 (hepatocellular), and A431 (epidermoid). The concentration of chimeric toxin that inhibited protein synthesis by 50% (EC50) was 110, 70, and 18 ng/mL for bFGF-PE40 and 15, 1, and 18 ng/mL for bFGF-PE4E KDEL. In comparison with fusion-toxins composed of acidic fibroblast growth factor (aFGF) and either PE40 or PE4EKDEL, bFGF-PE40 and bFGF-PE4E KDEL were similarly cytotoxic on most cell lines tested. Human aortic smooth muscle cells were sensitive to both bFGF and aFGF toxin fusion proteins. However, human aortic endothelial cells were sensitive to the bFGF-toxins but were resistant to both aFGF-toxin forms. Time course studies showed that bFGF-PE40 needed a 4-6-h exposure to target cells for peak inhibition of protein synthesis on both MCF-7 and A431 cells, while aFGF-PE40 was almost fully active within a 2-h incubation.(ABSTRACT TRUNCATED AT 250 WORDS)

MeSH

Amino Acid Sequence; Bacterial Toxins; Base Sequence; Chimeric Proteins; Comparative Study; DNA, Complementary; Endothelium, Vascular; Enzyme-Linked Immunosorbent Assay; Escherichia coli; Exotoxins; Fibroblast Growth Factor, Acidic; Fibroblast Growth Factor, Basic; Gene Expression; Heparin; Human; Molecular Sequence Data; Muscle, Smooth, Vascular; Neoplasms; Plasmids; Receptors, Fibroblast Growth Factor; Time Factors; Tumor Cells, Cultured

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Reiter

Medline

Cytotoxic and antitumor activity of a recombinant immunotoxin composed of disulfide-stabilized anti-Tac Fv fragment and truncated Pseudomonas exotoxin.

Reiter Y, Kreitman RJ, Brinkmann U, Pastan I

Int J Cancer 1994 Jul 58:142-9

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Abstract

Disulfide-stabilized Fv (dsFv)-immunotoxins are recombinant immunotoxins in which the inherently unstable Fv moiety, composed of the VH-VL heterodimer, is stabilized by a disulfide bond engineered between structurally conserved framework positions of VH and VL. Anti-Tac (dsFv)-PE38KDEL is composed of such a dsFv, directed to the alpha subunit of the IL2 receptor (IL2R), and containing a truncated form of Pseudomonas exotoxin (PE38KDEL). We have found this new type of immunotoxin to be indistinguishable in its in vitro activity and specificity from its single-chain immunotoxin counterpart, anti-Tac(Fv)-PE38KDEL. We have now examined the therapeutically relevant factors, including stability, pharmacokinetics, and antitumor activity of this new disulfide-stabilized Fv-immunotoxin. We found that anti-Tac(dsFv)-PE38KDEL was specifically cytotoxic to human activated T-lymphocytes in addition to IL2R bearing cell lines. Anti-Tac(dsFv)-PE38KDEL was considerably more stable at 37 degrees C in human serum and in buffered saline than the single-chain immunotoxin, anti-Tac(Fv)-PE38KDEL. The half-life in blood was similar for both immunotoxins (approx. 20 min). The therapeutic potential of the disulfide-stabilized immunotoxin was evaluated using an animal model of immunodeficient mice bearing subcutaneous tumor xenografts of human IL2R-bearing cells. Anti-Tac(dsFv)-PE38KDEL caused complete regression of tumors with no toxic effects in mice. Because dsFv-immunotoxins are more stable and can be produced with significantly improved yields compared to scFv-immunotoxins, dsFv-immunotoxin may be more useful for therapeutic applications.

MeSH

Animal; Antineoplastic Agents; Comparative Study; Disulfides; Drug Stability; Exotoxins; Female; Human; Immunoglobulin Fragments; Immunoglobulin Variable Region; Immunotoxins; Mice; Mice, Inbred BALB C; Mice, Nude; Neoplasm Transplantation; Neoplasms, Experimental; Peptide Fragments; Plasmids; Receptors, Interleukin-2; Recombinant Proteins; Support, Non-U.S. Gov't

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Order Document

Kuan

Medline

Pseudomonas exotoxin A mutants. Replacement of surface exposed residues in domain II with cysteine residues that can be modified with polyethylene glycol in a site-specific manner.

Kuan CT, Wang QC, Pastan I

J Biol Chem 1994 Mar 269:7610-6

BROWSE : J Biol Chem • Volume 269 • Issue 10

VIEW : MEDLINE, full MEDLINE, related records

Abstract

Pseudomonas exotoxin A (PE) is a three-domain protein in which domain Ia is involved in recognition of receptors on eukaryotic target cells, domain II promotes translocation of PE into the cytosol, and domain III enzymatically ADP-ribosylates elongation factor 2. Modification of proteins with polyethylene glycol (PEG) has been shown to prolong circulating plasma lifetime and may reduce or eliminate immunogenicity. However, in the case of toxins, PEG may interfere with or block toxin activity. To investigate the effect of polyethylene glycolation on specific residues located on the surface of PE domain II, we substituted cysteine, for each of the five most exposed surface amino acids (H276, E282, N306, R313, and E327) in domain II. These cysteines can serve as unique sites for PEG modification. The PE-Cys proteins retained most of their cytotoxicity even when the free sulfhydryl group was blocked by 5,5'-dithiobis(nitrobenzoic acid) or glutathione. When the PE-Cys proteins were conjugated with ovalbumin using a cleavable disulfide linkage, cytotoxicity was retained, but it was lost with a non-cleavable thioether linkage. In contrast, cytotoxicity was maintained when PE-Cys mutants were coupled to 5- or 20-kDa mPEG, using either a disulfide or a thioether linkage. Unexpectedly in some cases, the thioether conjugate was more active than the disulfide linkage. Pharmacokinetic studies on one of the polyethylene-glycolated molecules (R313C) showed that the mean residence time (t 1/2) was prolonged to 72 min, compared to 20 min for unpolyethylene glycolated PE-Cys(R313C). These studies show it is possible to derivatize PE at specific residues in domain II, maintain significant cytotoxic activity, and alter pharmacokinetics. These studies also suggest that large mPEG molecules can be translocated to the cytosol while still attached to domain II of PE.

MeSH

Animal; Bacterial Toxins; Cells, Cultured; Cysteine; Exotoxins; Human; Mice; Mice, Inbred BALB C; Mutation; Polyethylene Glycols; Protein Folding; Pseudomonas aeruginosa; Tumor Cells, Cultured

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Theuer

Medline

The N-terminal region of the 37-kDa translocated fragment of Pseudomonas exotoxin A aborts translocation by promoting its own export after microsomal membrane insertion.

Theuer CP, Buchner J, FitzGerald D, Pastan I

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VIEW : MEDLINE, full MEDLINE, related records

Abstract

The 37-kDa C-terminal fragment of Pseudomonas exotoxin A (PE; termed PE37 and composed of aa 280-613 of PE) translocates to the cell cytosol to cause cell death. PE37 requires a C-terminal endoplasmic reticulum retention sequence to be cytotoxic, indicating that the toxin may translocate to the cytosol from the endoplasmic reticulum. We show here that the N-terminal region of nascent PE37 can be inserted into the membrane of canine pancreatic microsomes by the preprocecropin signal sequence but then is exported or released from microsomes. The 34 N-terminal amino acids of the toxin fragment are sufficient to arrest translocation and prevent the microsomal accumulation of nascent chains that otherwise are sequestered into microsomes. These data support a role for the N-terminal region of PE37 in the translocation of the toxin from the endoplasmic reticulum to the cytosol in mammalian cells.

MeSH

Amino Acid Sequence; Animal; Cell Death; Cytosol; Dogs; Endoplasmic Reticulum; Exotoxins; Microsomes; Molecular Sequence Data; Mutagenesis, Site-Directed; Oligopeptides; Pancreas; Peptide Fragments; Plasmids; Polymerase Chain Reaction; Pseudomonas aeruginosa; RNA, Messenger; Restriction Mapping; Templates; Translation, Genetic

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Medline

Domain II of Pseudomonas exotoxin A arrests the transfer of translocating nascent chains into mammalian microsomes.

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Abstract

The translocation of PE from the extracytosolic compartment to the cytosol during the intoxication of mammalian cells is mediated by domain II of the toxin. We have shown previously that within domain II amino acids 280-313 of PE promote their own export from mammalian microsomes following signal sequence-directed membrane insertion. In this study, we attempted to target full-length PE into mammalian microsomes using the preprocecropin signal sequence, but found that translocation was arrested to generate a transmembrane protein. "Stop transfer" required the presence of amino acids 280-313 of PE, and the first 313 amino acids of PE were sufficient to generate a transmembrane protein (N-terminus-in/C-terminus-out). The mechanism of stop transfer appears to be different from that described previously because amino acids 280-313 of PE are not highly hydrophobic and do contain many charged residues. In addition, the transmembrane segment appeared to be influenced by the cytoplasmic domain of the transmembrane proteins.

MeSH

Amino Acid Sequence; Amino Acids; Animal; Bacterial Toxins; Base Sequence; Biological Transport; Dogs; Exotoxins; In Vitro; Microsomes; Molecular Sequence Data; Oligodeoxyribonucleotides; Protein Precursors; Protein Sorting Signals; Support, Non-U.S. Gov't; Support, U.S. Gov't, P.H.S.

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